Pathogenomic characterisation of a novel, layer-associated Avian Pathogenic *Escherichia coli*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by Charlotte Rose Collingwood

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the University's Regulations and Code of Practice for Research. Except where indicated, the work is my own. Work done in collaboration, or with the assistance of others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed: Date:

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3770 genome assembly and phylogenetic tree creation was performed by Dr Roy Chaudhuri, previously of the Centre for Genomic Research at the University of Liverpool, now Lecturer in Bioinformatics at the University of Sheffield.

Necropsies were performed with assistance from Dr Maria Camilla Brena, previously a Research Assistant in Infection Biology, University of Liverpool.

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List of Abbreviations

APEC: Avian Pathogenic *Escherichia coli*

EA/1: ExPEC Adhesin 1

ETT2: *E. coli* TTSS 2

ExPEC: Extra-Intestinal Pathogenic *Escherichia coli*

IL: Interleukin

kb: kilobase

LB: Luria Bertani

LPS: Lipopolysaccharide

Mb: Megabase

MHC: Major Histocompatibility Complex

MLEE: Multi-Locus Enzyme Electrophoresis

MLST: Multi-Locus Sequence Typing

MOI: Multiplicity of Infection

NGS: Next Generation Sequencing

NMEC: Neonatal Meningitis-associated *Escherichia coli*

OD: Optical Density

ORFs: Open Reading Frames

PAMPs: Pathogen Associated Molecular Patterns

PCR: Polymerase Chain Reaction

PPE: Personal Protective Equipment

SPS: Salpingitis-Peritonitis-Salpingoperitonitis

ST: Sequence Type

T3SS: Type III Secretion System

TLR: Toll-Like Receptor

UK: United Kingdom

UPEC: Uropathogenic *Escherichia coli*

USA: United States of America

UTI: Urinary Tract Infection

Abstract

Charlotte Rose Collingwood - Pathogenomic characterisation of a novel, layer-associated Avian Pathogenic Escherichia coli

Avian Pathogenic *Escherichia coli* (APEC) is an important pathogen of the poultry industry, responsible for 43% of condemnations of broiler birds at slaughter and annual losses of between 1-8% of a laying flock. APEC is also a public health concern; consumption of poultry meat has been linked with urinary tract infections (UTI) in humans, and also as a reservoir of potential antimicrobial resistance genes.

In this study of strain 3770, a reproductive tract associated isolate of APEC, the isolate was characterised for typical APEC virulence phenotypes, and also underwent full-genome sequencing in order to further advance our understanding of the pathogenomics of reproductive tract associated APEC infections. Additionally, a population study examined the virulence gene profiles within a population of reproductive tract associated *E. coli,* with a particular focus on virulence factors associated with infections in avian species and UTI in humans.

It was found that 3770 exhibited characteristic extra-intestinal pathogenic *E. coli* (ExPEC) virulence phenotypes; serum resistant, adhesive, and able to persist in the short term within phagocytic cells. It was also able to induce reproductive tract infections via the aerosol route. The genome sequence was 5.02Mb in size with a high number of virulence genes. The most closely related *E. coli* was an adherent-invasive *E. coli* isolate associated with Crohn's Disease. The population study of reproductive tract infections revealed a high level of variance within the population, and a higher prevalence of *ibeA* and K1 capsule genes than seen in other populations.

These results provide further evidence that there is no one single APEC pathotype. It is likely that virulence in APEC isolates is a complex relationship between the virulence profile of the bacterium and the health status of the host.

1.0 - Introduction

Avian Pathogenic *Escherichia coli* (APEC) is a substantial burden on the poultry industry, responsible for significant morbidity and mortality and subsequent economic losses. It also represents a potentially significant risk to public health as a source of zoonotic infection and also as a source of antibiotic resistance and other virulence genes.

APEC infections affect many domesticated avian species, including chickens (laying, broiler and broiler-breeder), turkeys, farmed partridge and quail (Dho-Moulin and Fairbrother 1999; Burns et al. 2003; Díaz-Sánchez et al. 2013) and it has also been observed in wild birds such as hen harriers and starlings (Gaukler et al. 2009; Vaughan-Higgins et al. 2013) which may serve as transmission vectors for free-range hens.

Annually, the UK places 918 million broiler chicks on farms (Crane et al. 2012) and poultry meat production is worth £1.89billion. Additionally, the UK egg production industry is valued at £559million. The overall rate of condemnation at slaughter for broilers is 1%, of which 43% are for reasons related to APEC infections (Yogaratnam 1995).

APEC also has substantial impact on layer flocks with between 1-8% of a flock can be lost to salpingitis annually (Cumming 2001) and egg production can drop by 2-3% in flocks affected by APEC (Morley and Thomson 1984). It is also thought that APEC infections contribute significantly to early broiler mortalities (Kemmett et al. 2014).

1.1 – *Escherichia coli*: the organism

Escherichia coli is a Gram-negative, motile rod-shaped facultative anaerobe in the family *Enterobacteriaceae*.

E. coli is found as part of the normal intestinal microflora, it resides in the mucus layer in the colon and caecum regions of the large intestine. Colonization occurs within hours of birth, not just in humans but in all warm-blooded animals and some reptiles (Smith 1965). Whilst *E. coli* are able to reside in water and sediment, their presence is usually indicative of faecal contamination of the environment (Tenaillon et al. 2010).

E. coli was one of the first bacteria to undergo whole genome sequencing, with the full sequence being published in *Science* in 1997 (Blattner et al. 1997). It is often used as a model organism, as a laboratory workhorse for recombinant DNA technology, and in biotechnology for production of proteins due to its well characterised growth kinetics including simple nutritional requirements and a rapid and replicable doubling time of around 20 minutes (Borriello et al. 2005; Sezonov et al. 2007) .

1.2 – *Escherichia coli*: the pathogen

E. coli has been known as a pathogen for some time. For over a century we have known of its links with various diseases of humans and domesticated animals. It was first demonstrated to be a causative agent of a disease in fowl by Lignières in 1894 (Sojka 1965) and but it wasn't until 1945 that it was also implicated in disease of humans (Bray 1945).

E. coli as a species encompasses many different sub-types capable of inflicting a wide variety of disease syndromes, with an overview of the infections possible in human hosts shown below in Figure 1.

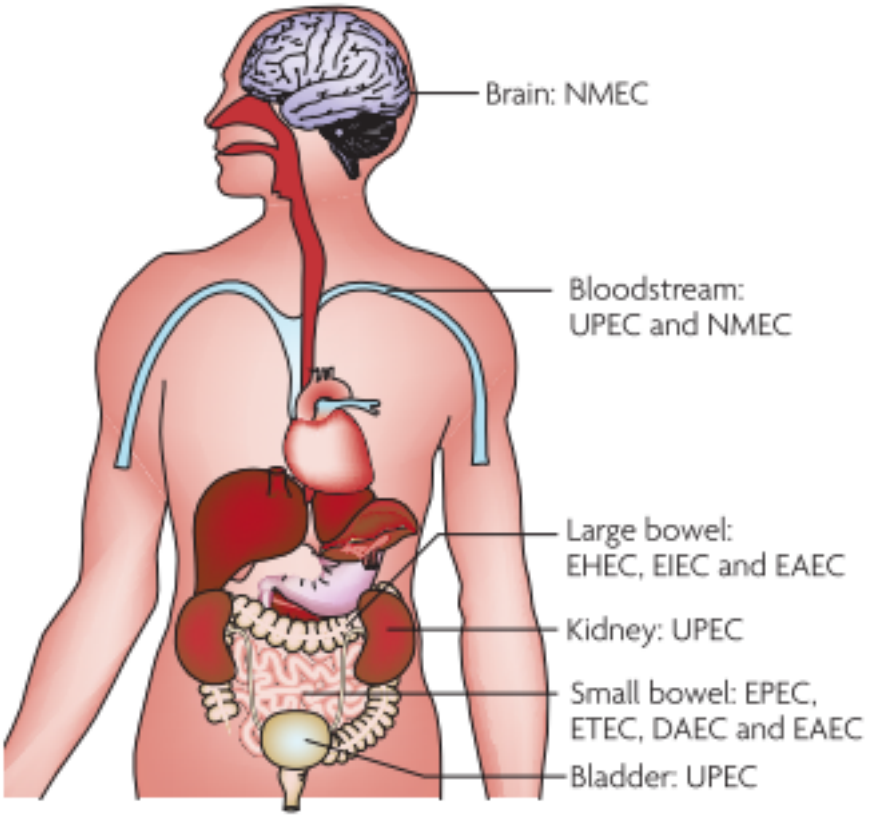

Figure 1: Human sites of E. coli infection

(Croxen and Finlay 2010). Neonatal Meningitis *E. coli* (NMEC), Urinary Pathogenic *E.* coli (UPEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroinvasie *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E.* coli (EPEC), Enterotoxigenic *E.* coli(ETEC), Diffusely-Adherent *E. coli* (DAEC)

The two main sub-types are those *E. coli* that cause disease within the gut (EPEC) and those that cause disease outside the gut such as UPEC and NMEC : extra-intestinal Pathogenic *E. coli* (ExPEC) (Nataro and Kaper 1998; Russo and Johnson 2000)

Enteropathogenic *E. coli* are the most well known sub-type, infections typically occur in outbreaks and present as serious gastrointestinal illnesses especially in children and the elderly (Abba et al. 2009). The World Health Organisation estimated in 2000 that diarrhoea illness accounted for 3.6% of global deaths, Figure 2 shows the global mortality rates for children under 5 in 2010 (Mathers et al. 2002; Croxen et al. 2013). Some pathotypes such as enteropathogenic *E. coli* are gut commensals in ruminant species, and this is recognised as the natural reservoir and most important source of human infections (Caprioli et al. 2005).

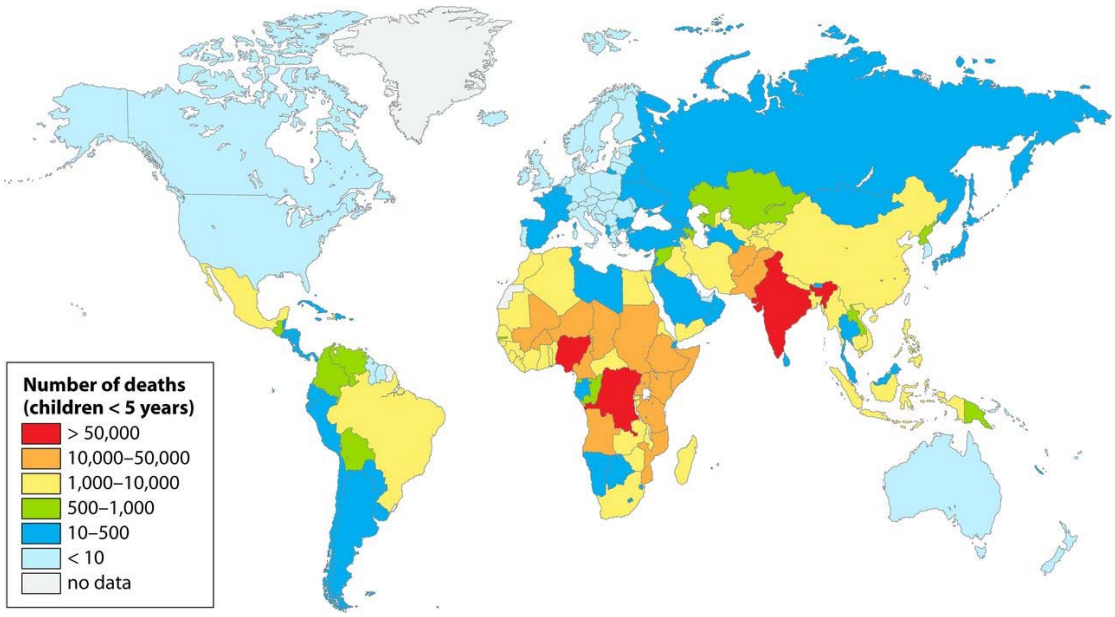

Figure 2: Global mortality in 2010 for children under 5 from diarrheal disease

(Croxen et al. 2013)

In contrast, ExPEC do not occur in outbreaks in humans, and as such there are no sensational news articles or public health announcements and consequently public awareness of ExPEC is low. They are able to efficiently colonise the gut of

the host prior to causing extra-intestinal infection, and many of the diseases caused are minor or affect already immune-compromised hosts (Johnson and Russo 2002; Russo and Johnson 2003; Kaper et al. 2004).

1.2.1 – Extra-Intestinal Pathogenic *Escherichia coli*

ExPEC are able to live harmlessly in the gut environment of the host, only causing disease when the bacteria leave the environment of the gut and enter an extra-intestinal site such as the urinary, reproductive tract or respiratory tract. This can happen if the gut wall is perforated, if the bacteria are aerosolized and inhaled, if the bacteria ascend the urinary tract, or via translocation in stressed birds (Dominick and Jensen 1984).

The most commonly described ExPEC infections are neonatal meningitis, septicaemia and urinary tract infections in humans and colibacillosis in chickens. They cause a high economic burden, both for the poultry industry and in terms of human health care costs and loss of productivity (Yogaratnam 1995; Pitout 2012).

ExPEC frequently belong to the B2 and D phylogenetic groups (as defined using multilocus enzyme electrophoresis), whereas EPEC typically belong to groups A, B1 or D and commensal *E. coli* are usually in groups A and B1 (Johnson and Russo 2002).

1.2.1.1 – ExPEC as a public health concern

Extra-intestinal pathogenic *E. coli* frequently live quite harmlessly in the gastrointestinal tracts of their hosts without causing disease. Indeed, it is possible that they are more successful at colonizing the gastrointestinal tract than non-pathogenic faecal *E. coli* – it has been suggested that they are the predominant strain of *E. coli* in the gastrointestinal tract of around 20% of normal individuals (Johnson 1991). ExPEC infections in humans are known to be an important public health concern. *E. coli* are responsible for millions of urinary

tract infections (UTI) annually worldwide, which cause considerable financial losses due to lost productivity and increased healthcare costs. *E. coli* are also a frequent causative agent of neonatal meningitis and sepsis, both of which cause serious sequelae and death, not to mention significant distress (Russo and Johnson 2003).

An additional aspect of ExPEC epidemiology is the possibility that the bacteria are able to move from animal to human hosts via the food chain. It has been shown that following preparation of raw poultry meat for cooking, bacteria contaminating the surface of the chicken can colonise the host gastrointestinal tract and be excreted for up to 10 days post-preparation (Linton et al. 1977).

Numerous studies have observed ExPEC contamination of retail meats, particularly poultry, in high numbers (Johnson et al. 2005a, b, 2009; Jakobsen et al. 2010; Xia et al. 2011; Bergeron et al. 2012). Furthermore, consumption of poultry meat has been linked to antimicrobial resistant urinary tract infections in women. Studies in both Denmark and Canada have shown that strains isolated from UTI in women have strong similarities with strains isolated from local meat and production animals (Manges et al. 2007; Jakobsen et al. 2010, 2012).

Collectively, these studies provide strong evidence that human ExPEC infections have an animal reservoir, and that animal ExPEC isolates can be considered a zoonotic risk. Thus, APEC infections are not just a concern for the poultry industry but also from a public health standpoint.

Additionally, as ExPEC are able to live harmlessly in the gastrointestinal tract, there could be a long period of time between acquisition of the bacteria and an event that allows extra-intestinal disease to occur. As it is difficult to determine the original source of infection; it is likely that zoonotic infections from APEC are significantly underestimated.

1.2.2 – Avian pathogenic *Escherichia coli*

APEC is an important pathogen of the poultry industry, causing significant morbidity and mortality and subsequent economic losses. The annual burden on the poultry industry is high, lesions consistent with colisepticaemia are responsible for 43% of broiler carcass condemnations and it has been estimated that the overall cost of condemnations on the UK broiler industry is £16.5 million per annum (Yogaratnam 1995) thus APEC infections could be responsible for losses of around £7.1 million. However this figure is likely to be a conservative estimate given that it does not consider the burden on breeder and layer facilities and also that more broiler chicks are placed on farms currently than in 1995 (Crane et al. 2012). Furthermore there is anecdotal evidence that removal of growth promoting antimicrobials in the EU, prior to and since the ban on their use came into force in 2006, has lead to an increase in bacterial disease largely manifesting as necrotic enteritis or colibacillosis.

In laying hens, between 1-8% of a flock each year can be lost due to infections of the reproductive tract (Cumming 2001) which include salpingitis, peritonitis and salpingoperitonitis (SPS) (Jordan et al. 2005). Previously, it has been poorly understood the reasons by which APEC is able to cause reproductive tract infections in laying hens, however it is thought to originate in one of two ways. The first theorised route is via the respiratory tract; following inhalation, the bacteria migrate from the left airsac into the ovaries and progress to infect the reproductive tract (Dho-Moulin and Fairbrother 1999). The second theorised route is bacterial ascension of the vent after pecking injuries from cage-mates (Cumming 2001).

Whilst the route of infection remains to be clearly determined, the original source of bacteria is relatively certain. It has previously been estimated that up to 15% of the coliforms resident in the chicken gastrointestinal tract belong to potentially pathogenic serotypes (Harry and Hemsley 1965) which is close to the

estimate of by Johnson of 20% for potentially-pathogenic *E. coli* in the human gastrointestinal tract (Johnson 1991).

Both virulent and avirulent strains of *E. coli* have been isolated from the intestinal tract of turkeys and it was shown that when the birds were stressed, the bacteria was able to translocate to extra-intestinal sites (Dominick and Jensen 1984). Zanella et al. identified septicaemia of laying broiler breeders at point of lay (Zanella et al. 2000), which has been shown elsewhere to be a time of immunosuppression for the maturing hen (Johnston et al. 2012)

More recently, longitudinal studies of broiler chickens from placement to slaughter observed potentially pathogenic isolates of *E. coli* in the gut of otherwise healthy birds – these were defined as *E. coli* with more than 5 virulence-associated genes (Kemmett et al. 2014).

Finally, over the past 50 years there have been a number of studies on the level of aerosolized bacteria present in the hen house, focusing on both aerosolized bacteria as a problem for health of the chickens resident in the houses (Oyetunde et al. 1978), as a problem for poultry farm workers (Whyte 1993; Donham et al. 2000; Kirychuk et al. 2006) and as a potential environmental concern (Bakutis et al. 2004). These studies have all observed high levels of aerosolized bacteria present. These aerosolized bacteria could serve as a source of infections not only for the birds inside the hen house but also those human workers who do not always wear respiratory personal protection equipment (PPE).

1.2.2.1 – APEC disease manifestation

E. coli infections of birds have different presentations and severities depending on the age, production type, and health of the bird. All infections begin with APEC entering host tissues; this could be through an injury, or following colonisation of a mucosal surface. Type 1 fimbriae are thought to be important

for this initial attachment phase; they have been shown to adhere to epithelial cells in the upper trachea and oviduct (Pourbakhsh et al. 1997b; Monroy et al. 2005), and also to mucosal surfaces in the digestive tract (Edelman et al. 2003).

Entrance into the tissues stimulates the host immune response and production of the pro-inflammatory cytokines interleukin (IL) 1, and IL-8 (Chamanza et al. 1999). IL-1 induces pyrexia and T cell proliferation, IL-6 causes up-regulation of acute phase proteins such as transferrin and α_1-acid glycoprotein (Chamanza et al. 1999). Heterophils release β-defensins, which kill the bacteria, but form exudate, a cheese-like mass of dead, and dying bacteria, heterophils and heterophil granulate. Later on in the infection, phagocytic cells arrive to break down the exudate however by this point tissue damage can have already occurred leaving fibrous lesions and scarring if the bird recovers. Lesions occur more frequently in infections with less virulent isolates, when the progression of infection is very quick; the bird dies before lesions are able to form.

Newly hatched chicks and embryos often suffer from yolk sac infections, omphalitis and colisepticaemia (Iqbal et al. 2006). It is thought that infections in chicks may occur *in ovo via* contamination of the surface and subsequent penetration of the egg during incubation (yolk sac infection), or post-hatching *via* inhalation of aerosolized bacteria and subsequent colonisation of the respiratory tract or infection of the unhealed naval (omphalitis). It is estimated that between 0.5-6% of eggs laid by healthy hens contain APEC inside the egg (Barnes et al. 2008), however as Barnes also mentions, it has been shown experimentally that hens recovered from APEC infection remain persistently colonized for up to 21 weeks post infection, and of their eggs, 2.7% contain *E. coli* (Barnes et al. 2008), it is possible that the number of APEC-containing eggs laid by 'healthy' hens unknowingly also includes eggs laid by recovered and colonized birds.

Broiler-breeders and layers are more prone to reproductive tract infections such as salpingitis and peritonitis. This can present as a low-level colonisation of the

oviduct, where the hens remain in lay but egg production decreases and the birds appear depressed, or more serious infection, spreading throughout the abdomen *via* the oviduct, resulting in death. It has been suggested that high egg production is a risk factor for SPS infections, whereby over-relaxation of the uterovaginal sphincter allows bacteria to ascend to and colonise the oviduct (Landman et al. 2013).

Broilers typically suffer from colibacillosis originating from respiratory tract infections. This begins as airsacculitis, and can spread to other organs resulting in pericarditis and perihepatitis. If the bacteria enter the blood stream, septicaemia occurs which is usually fatal. APEC infections in broilers can also present as cellulitis and necrotic dermatitis. Whilst these are not fatal, they do result in the condemnation of the carcass at slaughter, thus they are economically important (Elfadil et al. 2014).

1.2.2.2 – APEC epidemiology

The prevalence of *E. coli* infections in poultry is high, in laying hens losses due to salpingitis can be between 1-8% annually (Cumming 2001) and mortality in newly hatched layer-chicks are often attributed to APEC infections. APEC outbreaks in broilers result in mortality rates of up to 20% of the flock, and birds who do not show obvious signs of infection may still go on to be condemned at slaughter (Yogaratnam 1995).

It was previously thought that *E.* coli infections in chickens occurred after primary infection with a virus such as Newcastle disease virus or a mycoplasma (Dho-Moulin and Fairbrother 1999), however it is now recognized that APEC infections can be primary infections (Cheville and Arp 1978; Dhillon and Jack 1996; Zanella et al. 2000). There are many predisposing factors linked to APEC infections. These include stress, exposure to ammonia and dust, contaminated water, modern fast-growing breeds, and trauma (Barnes et al. 2008). Thus, to

some extent, control of colibacillosis is possible on a farm level by reduction of these predisposing factors.

As well as the *E. coli* already resident in the gut population of the flock, there are transmission vectors that allow new strains of *E. coli* to colonise the gut microbiota of the flock. For caged and barn-housed birds, insects such as darkling beetles and houseflies are the most common vector (Barnes et al. 2008). For free-range birds, wild birds such as starlings (Gaukler et al. 2009), and waterfowl such as ducks and geese (Fallacara et al. 2001; Cole et al. 2005) are a risk factor for new strains of APEC. These sources are able to introduce new, possibly more virulent, strains of *E. coli* into the hens' environment, and also serve as a pool for virulence genes and antibiotic resistance.

1.2.3 – Urinary tract pathogenic *Escherichia coli*

UTI are a common infection of humans, particularly women, with approximately 40% of women and 12% of men experiencing symptomatic UTI during their lifetime. Of these UTI, most are caused by uropathogenic *Escherichia coli* (UPEC). UTI are estimated to account for 2-40% of nosocomial infections (Foxman 2002; Bjerklund Johansen et al. 2006). It is estimated that the cost of UTI in the USA annually exceeds $3 billion (Sivick and Mobley 2010). The classification of a UTI is dependent upon the location of the infection: cystitis within the bladder, pyelonephritis within the kidney, and bacteriuria within the urine.

UPEC infections are thought to originate in the gut flora of the individual, entering the urinary tract *via* the faecal-vaginal-urethral route (Gruneberg 1969; Russo et al. 1995; Yamamoto et al. 1997). As mentioned earlier, it has also been suggested that these gut bacteria originate from consumption of retail meat and that they are in fact a zoonosis (Bergeron et al. 2012; Manges and Johnson 2012).

After *E. coli* enter the urinary tract, the adhesive FimH tip of type 1 fimbriae facilitates adhesion to the urinary epithelium. Although type 1 fimbriae are seen to initiate adhesion, as required for invasion, no difference in expression of type 1 fimbriae are seen across pathogenic or commensal strains (Hagberg et al. 1981). FimH binds to uroplakins and integrins on the uroepithelial surface causing internalization of the bacteria via actin rearrangement.

P fimbriae are associated with ascending UTIs and pyelonephritis (Plos et al. 1995). P fimbriae bind to glycolipids present on the surface of renal epithelia; the attachment of P fimbriae elicits an immune response induced *via* Toll like receptor 4 signalling pathway (Fischer et al. 2007). If the bacteria are able to ascend to the kidneys, there is potential for damage to the kidneys to occur; severe infections can cause renal failure (Ronald 2002).

Once inside the cells, UPEC replicate and form biofilm-like pods (intracellular bacteria communities: IBCs). IBCs are a protective environment; the bacteria within are protected from the host immune system and antibiotics (Hvidberg et al. 2000). UPEC leave these communities sporadically, causing reinfection, thus many patients suffer recurrent UTI with the same strain (Anderson et al. 2010). IBC cannot be detected with confidence in urine samples, due to the low exfoliation rate of urinary tract epithelial cells no bacteria may be detected in the urine sample.

Damage to the urinary tract during UTI results from exfoliation of uroepithelial cells as a host response to UPEC attachment and invasion, whilst the influx of white blood cells in response to bacteria causes inflammation.

1.3 – Virulence factors of *Escherichia coli*

There are many genes in ExPEC that have been associated with virulence. These include iron acquisition systems, factors to evade host defence proteins, and cell-surface structures. Virulence factors are often found on plasmids and pathogenicity islands and these are frequently acquired horizontally from related bacteria. There are two main virulence plasmids in APEC, these are the pAPEC-O1 and ColV (and the closely related ColB) plasmids, these will be discussed in section 1.4.4.

Many virulence genes are considered necessary for virulence, and knockout mutants will show reduced ability to infect a host. However, not all pathogenic strains contain all of these potential virulence genes, and faecal *E. coli* of healthy hosts sometimes possess many virulence factors. We have much to learn still on what causes pathogenicity in ExPEC.

1.3.1 – Adhesins and lipopolysaccharide

Bacterial adhesins are surface structures that are produced by bacteria in order for them to adhere to host epithelial cells or other surfaces in their environment and prevent their removal from those surfaces. Adhesins interact with specific receptors, thus the presence of an adhesin does not mean that the bacteria will be able to adhere to all surfaces (Klemm et al. 2010).

Adhesins of APEC include the type 1 fimbriae, P or Pap fimbriae, Curli fibres, S fimbriae, ExPEC Adhesin 1, afimbrial adhesins and the temperature sensitive haemagglutinin.

1.3.1.1 – Flagella

Flagella are multiprotein assemblies that project 15-20µm from the bacterial cell surface, which are known to be important for motility and chemotaxis (Silverman and Simon 1977). In addition, more recent evidence has suggested that in certain contexts flagella are also able to act as adhesins and immune modulators (Rossez et al. 2015).

Flagella are encoded for by large gene clusters termed 'flagellar regions I, II, IIIa and IIIb' in which many genes encode for structural proteins (region I), chemotaxis genes (region II), flagellar filament (region IIIa) and early flagellar assembly (region IIIb).

Evidence in avian tissue culture and tracheal explants has suggested a role for flagella in allowing bacteria to penetrate through a protective mucus layer allowing the bacterial to make contact with the host epithelia, however in the absence of mucus no functional disadvantage was observed in the flagella mutant strain (La Ragione et al. 2000a).

1.3.1.2 – Type 1 Fimbriae

Type 1 fimbriae are mannose-dependent fimbriae that bind mannose-containing receptors found on the host cell membranes. Type 1 fimbriae are found in the majority of ExPEC and are encoded for by the fimABCD operon. The expression of type 1 fimbriae is phase-variable, under control of *fimB* and *fimE* (Klemm et al. 2010).

Type 1 fimbriae have been shown to be involved in pathogenicity of APEC infections; their presence on the infective strain increased the number of chicks developing disease and also the severity of the disease (Naveh et al. 1984). Evidence suggests that type 1 fimbriae play a role in the early stages of

infection, as they are expressed by bacteria during colonisation of the trachea, air sacs and lungs, but not blood or other systemic organs (Pourbakhsh et al. 1997b), however it is possible that there is functional redundancy as one lab has shown that their *fim*$^{-}$ mutants are able to colonise the respiratory tract as well or better than wild type (Marc et al. 1998; Arné et al. 2000). In UPEC, the adhesive tip, FimH, has been observed to mediate biofilm formation, and may be important for invasion of bladder epithelial cells (Mulvey et al. 1998).

1.3.1.3 – P Fimbriae

P fimbriae, also known as pap (**p**yelonephritis **a**ssociated **p**ili) bind to host cells *via* an adhesive tip, *papG,* for which there are three different variants within ExPEC populations, each binding a different receptor (Kariyawasam and Nolan 2011). Pap fimbriae have been recognized as a virulence factor for ExPEC (Smith et al. 2007) however their role in pathogenicity is controversial.

Expression of P fimbriae during infections of chicks has been localized to bacteria in the air sacs, lungs, kidney, blood and pericardial fluid, which was interpreted as indicating P fimbriae play a role in the later stages of infection (Pourbakhsh et al. 1997b), however, other evidence suggests that P fimbriae are non-essential for virulence. They are frequently observed in only around 30% of APEC populations (Dozois et al. 1992; Janßen et al. 2001; Johnson et al. 2001a; Delicato et al. 2003; Kemmett et al. 2014) and *pap*$^{-}$ mutants have been shown to be no different to wild type in terms of pathogenicity in chick models (Stordeur et al. 2004).

Additionally, strains possessing the operon may not express P fimbriae (Dozois et al. 1992), whilst others may possess only part of the operon (Kariyawasam and Nolan 2011). Thus evidence suggests that whilst P fimbriae may play a role in infection, they are non-essential and there may be some functional redundancy with other adhesins.

1.3.1.4 – Curli Fimbriae

Curli fimbriae are encoded for by two operons, *csgBAC* and *csgDEG*. (Gophna et al. 2001) and are thought to be involved in virulence of ExPEC *via* interactions with Class I Major Histocompatibility Complex (MHC) found on most mammalian cell types (Olsén et al. 1998), or through interactions with matrix and plasma proteins (Antão et al. 2009). They may also play a role in internalization of bacteria (Gophna et al. 2001) and have been shown to be involved in adhesion to avian gut explants (La Ragione et al. 2000a).

Curli-encoding genes are often ubiquitous in APEC and avian faecal *E. coli* populations (Olsen et al. 1989; Maurer et al. 1998; Dho-Moulin and Fairbrother 1999; Delicato et al. 2003; McPeake et al. 2005) however bacteria possessing the genes may not always be able to express the curli phenotype (Maurer et al. 1998). Despite this, it seems likely that curli fibres are important for *E. coli* due to their high prevalence in both healthy and disease-associated isolates.

1.3.1.5 – S Fimbriae

S fimbriae are encoded for by *sfaAGHS* and regulated by *sfaBC* (Antão et al. 2009b). They bind to neuraminic acid (Wright and Hultgren 2006) and are thought to be involved in crossing the blood brain barrier (Korhonen et al. 1985) and are also able to adhere to human urinary tract epithelial cells suggesting a role in UTI (Korhonen et al. 1986).

As of yet, no role has been suggested for S fimbriae in APEC infections, however the gene does occur sporadically in APEC populations (Rodriguez-Siek et al. 2005a; McPeake et al. 2005; Ewers et al. 2007; Timothy et al. 2008) at between 5-10% prevalence. Thus it may be able to play a role in infections of avian species, and may also present a zoonotic risk to human hosts.

1.3.1.6 – ExPEC Adhesin 1

ExPEC adhesin 1 (EA/1) is a recently discovered adhesin of ExPEC, encoded for by the putative *yqi* gene cluster, identified using signature-tagged mutagenesis. Deletion caused attenuation of the strain both *in vivo* and *in vitro, which* was returned when the gene was complemented. Electron microscopy revealed that the gene encodes for short, fimbrial-like appendages. A study of 900 *E. coli* isolates (ExPEC, intestinal pathogenic and non-pathogenic *E. coli*) showed that possession of the gene was strongly associated with ExPEC populations, with a prevalence of around 60% in APEC, UPEC and neonatal meningitis-associated *E. coli* (NMEC) isolates. So far no receptor for this adhesin has been identified, however studies of *csq* APEC mutant strains have reduced ability to colonise the avian lung, or gut cells (La Ragione et al. 2000a; Antão et al. 2009b). Curli fimbriae are not critical to APEC virulence but are suggested to increase adhesion to lung and gut cells, lowering the required infectious does to cause infection.

1.3.1.7 – Afimbrial Adhesins

Afimbrial adhesins are truncated fimbriae, encoded for by the *afaABCDE* operon, of which there are several variants encoding for different adhesins (Antão et al. 2009b; Mainil 2013). They are less commonly seen in APEC populations than other adhesins; when the gene is seen in a population, prevalence is between 1.3-12.5% (Stordeur et al. 2002; Amabile de Campos et al. 2005; Ewers et al. 2007; Johnson et al. 2008a) however many studies do not find the presence of the gene at all (Dozois et al. 1992; Knöbl et al. 2004; McPeake et al. 2005; Johnson et al. 2008b; Frömmel et al. 2013).

Nevertheless, a study has shown that *pap*- isolates possessing *afa-8* genes isolates were lethal in a one-day-old chick model and able to cause colibacillosis when inoculated both *via* intra-tracheal and thoracic air sac (Stordeur et al.

2004) indicating that afimbrial adhesins may be involved in APEC pathogenesis in the absence of other adhesins.

1.3.1.8 – Temperature Sensitive Haemagglutinin

The **t**emperature **s**ensitive **h**aemagglutinin is encoded by the gene *tsh*. The gene is found on the ColV plasmid, in the variable region (Johnson and Nolan 2009). It was first observed in APEC strain χ7122, and was shown to cause mannose-resistant agglutination of chicken erythrocytes and have significant homology to immunoglobulin proteases found in *Haemophilus* and *Neisseria* species (Provence and Curtiss III 1994).

More recently, Kobayashi et al. have shown that Tsh has proteolytic activity against mucins, with both supernatant from APEC and recombinant Tsh causing proteolysis of chicken tracheal mucin suggesting a role in colonization and invasion of the chicken trachea in the early stages of infection (Kobayashi et al. 2010). Another study showed that Tsh caused high levels of virulence was involved in development of lesions in the airsacs but not further development of pericarditis, perihepatitis or septicaemia, also suggesting a role in the early stages of infection (Dozois et al. 2000). However a different study argues that *tsh* is not essential for virulence; in their study only one virulent isolate possessed *tsh*. They suggest that other adhesins could be substituted, suggesting a level of redundancy (Tivendale et al. 2004).

tsh prevalence in APEC populations is generally high, with figures suggesting prevalence of 46% (Maurer et al. 1998), 90.6% (Dho-Moulin and Fairbrother 1999), of pathogenic isolates. However its importance is not universally agreed upon, with another study finding it in only one strain of a small panel of 5 isolates (Tivendale et al. 2004), however this would still indicate a prevalence of 20% in the population assuming the 5 isolates were adequate representatives.

1.3.1.9 – Lipopolysaccharide

Lipopolysaccharide (LPS), also known as endotoxin, is vital for Gram-negative bacteria survival. The outer membrane surface of Gram-negative bacteria can be made up of 75% LPS and it plays a role in protecting the cell from hydrophobic substances (Alexander and Rietschel 2001).

LPS is encoded for by a number of genes, found in different locations on the chromosome. The inner and outer core region are encoded for by a locus named *waa* whereas the lipid A synthesis genes are found in a cluster named *lpxABCD* and synthesis genes are found in the cluster *wba* (Gronow and Brade 2001).

In addition to protecting the bacterial cell, LPS strongly stimulates both innate and acquired immune system of many eukaryote systems. The lipid A region of LPS activates TLR-4 which triggers subsequent inflammation (Raetz and Whitfield 2002).

1.3.2 – Metal Transport Systems

Metals such as iron, zinc, nickel and copper are essential for many cellular processes, however the concentration of these metal ions within the cytoplasm must be strictly regulated. If there is too low a concentration of a particular iron, certain metalloproteins and enzymes will be inactive, whilst too high a concentration will cause toxicity (Waldron et al. 2009). To ensure the correct concentration within the cytoplasm, bacteria must often scavenge metals from their surrounding environment. Iron concentrations are low in the host, as iron is sequestered into host proteins such as ferritin, haem or transferrin as a means to prevent bacterial growth *via* lack of nutrient availability (Weinberg 2009; Skaar 2010). Iron is involved in many metabolic processes, and is required for the function of numerous proteins. A key metalloprotein involved in *E. coli* virulence is the iron-containing protein superoxide dismutase which oxidises reactive oxygen species (Lah et al. 1995). For this reason, many bacteria have

multiple iron uptake systems; in APEC these are salmochelin, enterobactin, aerobactin, and the *sit* locus, which will be discussed in detail below.

1.3.2.1 - *sitABCD* locus

The *sitABCD* locus was first identified in 1999 in *Salmonella enterica* serovar Typhimurium (Zhou et al. 1999). It is an ATP binding cassette iron transport system and is often found on genomic islands or large plasmids in ExPEC; one study showed that it was present in 86.4% of APEC strains they tested, compared to only 42.7% of strains isolated from the faeces of healthy birds (Rodriguez-Siek et al. 2005a) - but it is not often seen in diarrheagenic strains of *E. coli*. This suggests that it may have a particular role in extra-intestinal infection. This suggestion is supported by evidence that the *sit* genes are up-regulated by the UPEC strain CFT073 during infection of the murine urinary tract and *in vitro* (Snyder et al. 2004) and also during infection of cells by the closely related bacterium *Shigella flexneri* (Lucchini et al. 2005).

The SitABCD transporter is also capable of transporting manganese and in fact this may be more important than its ability to scavenge iron. In conjunction with the manganese transporter *mntH* it was shown to increase resistance to oxidative stress in the avian pathogenic strain of *E. coli* χ7122, however on its own it does not (Sabri et al. 2006). Infection of mice by a *sitABCD* mutant strain exhibited a significant reduction in colonisation in the liver, lungs and spleen (Sabri et al. 2008). It is possible that Sit plays a more important role of acquiring manganese during infection of the host than the uptake of iron (Sabri et al. 2006).

1.3.2.2 – Enterobactin

Enterobactin was discovered concurrently in both *E. coli* and *Salmonella* species and is produced by nearly all *E. coli* strains (Garénaux et al. 2011). Enterobactin

is encoded for by a single gene cluster containing 15 biosynthesis and transport genes (Crosa and Walsh 2002). Studies using mutants of *E. coli* χ7122 in a chicken infection model have shown that enterobactin was functionally redundant when other siderophores were available (Caza et al. 2011).

1.3.2.3 - Aerobactin

The siderophore aerobactin is encoded by the *iucABCD* locus (**i**ron **u**ptake **c**helate), and the outer membrane protein receptor is encoded by *iutA* (**i**ron **u**ptake **t**ransport) (Warner et al. 1981). It was first isolated from the enterobacterium *Aerobacter aerogenes* (now known as *Enterobacter aerogenes*) grown in an iron-depleted environment (Gibson and Magrath 1969). It is also found in ExPEC, including APEC, where it is frequently found on the ColV and ColBM plasmids, but it is also sometimes integrated into the chromosome (Johnson et al. 2006c).

It has been shown using strain χ7122 in a chicken infection model that aerobactin is important for virulence in chickens, deletion was shown to reduce persistence and lesions in deep tissues (Dozois et al. 2003). Similarly, aerobactin was also shown to be important in a mouse model of urinary tract infection using strain CFT073, as deletion of the receptor *iutA* reduced the virulence of the mutant and it was out-competed by co-inoculated mutants without the deficiency (Garcia et al. 2011). Ling et al. reported that single mutants of *iutA*, *iucA* and *iucC* exhibited a significant reduced in survival levels during *in vivo* coinfection with a wild type strain (Ling et al. 2013).

Aerobactin is able to scavenge iron in serum, and very low concentrations of aerobactin (compared to enterobactin) are sufficient for growth. The additional capacity for iron uptake, alongside the other transporters could be advantageous (Sabri et al. 2008).

1.3.2.4 - Salmochelin

IroN is the receptor for the siderophore salmochelin, so named because it was discovered first in *Salmonella enterica* (Bäumler et al. 1998). Salmochelin is very similar to the siderophore enterobactin, with just minor differences in glycosylation. Enterobactin is easily sequestered by host factors such as neutrophil gelatinase-associated lipocalin in mammals, and a similar lipocalin has been tentatively identified in poultry (Coudevylle et al. 2011). The difference in glycosylation enables salmochelin to evade these host factors, and thus salmochelin is an important for growth of APEC in the host (Garénaux et al. 2011).

At first it was thought that *E. coli* did not possess the *iroBCEDN* locus, however it has been observed in some strains of ExPEC. In APEC, possession of this locus is associated with virulence. The *iroBCDEN* locus is frequently found on the ColBM or ColV plasmids (Johnson et al. 2006c, b) however it has also been observed on the APEC O1 plasmid (Sabri et al. 2006), and integrated into the chromosome (Dobrindt et al. 2002).

IroN is important for virulence in ExPEC, it has been shown to be overexpressed during infection of mouse uro-epithelial cells by the UPEC strain UTI189 (Reigstad et al. 2007), indicating that the salmochelin is essential in infection of the murine urinary tract. It has also been shown to be important for virulence in chicks in the APEC strain χ7122. A knockout mutant showed reduced virulence and indicated that the salmochelin is also important for persistence in deep tissues (Dozois et al. 2003). For this reason, *iroN* is sometimes as part of a gene panel in rapid diagnostic PCR to determine if a strain is virulent (Johnson et al. 2008b).

1.3.3 – Capsules & Self-Defence Mechanisms

Capsules are a protective layer of polysaccharides found on the cell surface of many bacteria. All members of *Enterobacteriaceae* are able to form capsules, and their composition differs by strain (Schembri et al. 2004). *E. coli* capsules either consist of K antigen, of which there are over 80 serotypes, or a polymer that closely resembles the O antigen, minus the terminal lipid A region (Whitfield and Roberts 1999).

It is thought that capsules serve many functions, including aiding adhesion and transmission of the bacteria, protecting the bacteria from the factors of the host immune system such as complement and white blood cells, increasing the bacteria's ability to survive intracellular environments, and protecting them from phages (Roberts 1996; Kim et al. 2003; Schembri et al. 2004).

Some capsule types have been shown to be important for virulence, such as the K1 capsule, which is comprised of sialic acid residues, also found on host surfaces thus enabling the bacteria to evade the immune system by molecular mimicry (Harvey et al. 2001). The K1 capsule has been implicated in both avian and human extra-intestinal infections (Brée et al. 1989; Mellata et al. 2003a; Kim et al. 2003; Ewers et al. 2007; Goller and Seed 2010; Barbieri et al. 2013) and is considered an important virulence factor for extra-intestinal *E. coli* (Dho-Moulin and Fairbrother 1999; Johnson and Russo 2002).

1.3.3.1 – Colicin

Colicin (*cva/cvi*) is a controversial, plasmid-encoded virulence factor of *E. coli* (Johnson and Nolan 2009), and researchers are divided as to its importance for pathogenicity. Early studies state clearly that colicin is not essential for virulence (Quackenbush and Falkow 1979; Williams and Warner 1980), and suggest that other genes on the plasmid are the virulence factors. However it is still included in some genetic studies of virulence factors of APEC populations in which its

high prevalence and correlation with pathogenicity are noted (Vidotto et al. 1990; Blanco et al. 1997; Dias da Silveira et al. 2002; Oh et al. 2011), others observed it less frequently or noted that there was no significant difference between faecal and pathogenic populations (Pfaff-McDonough et al. 2000; McPeake et al. 2005; Kemmett et al. 2014).

Whilst much research has been done on the prevalence of colicin and colicin genes in ExPEC populations, often researchers fail to consider work from population, evolutionary and molecular biologists. Extensive work on colicins has been carried out focusing on their function as antibiotics and also the high level of resistance to colicins in *E. coli* populations (Cascales et al. 2007). This evidence suggests that colicins in APEC populations play a role in outcompeting the host gut flora, rather than pathogenicity. However *cva/cvi* may remain useful markers for ColV/BM plasmids, which contain other important virulence factors.

1.3.3.2. - Increased Serum Survival

The *iss* (**I**ncreased **S**erum **S**urvival) gene was first discovered in a neonatal meningitis-causing *E. coli* (NMEC) strain, and it was observed that it conferred a 20-fold increase in resistance to serum and a 100-fold increase in virulence in a one-day-old chick model as compared to wild type strains (Binns et al. 1979, 1982; Chuba and Palchaudhuri 1986). Whilst little work has been done on the structure or action of Iss, the location of the gene is known to be in the constant region of the transmissible ColV/BM plasmids (Johnson and Nolan 2009) and a gene product has been cloned, monoclonal antibodies formed against the gene product (Horne et al. 2000; Foley et al. 2003) and the purified gene product has been shown to be immunogenic and a potential vaccine candidate in chickens (Lynne et al. 2006, 2012).

iss is highly prevalent in APEC populations, with most studies describing a prevalence of around 80% or higher in APEC disease-associated isolates (Pfaff-McDonough et al. 2000; Jeffrey et al. 2002; Rodriguez-Siek et al. 2005a; McPeake et al. 2005; Oh et al. 2011; Barbieri et al. 2013; Badouei et al. 2016) however some studies have seen significantly lower prevalence (Delicato et al. 2003; Kemmett et al. 2014). Finally, due to its strong correlation with pathogenicity in APEC, it is also used as a marker for virulence in two virulotyping multiplex polymerase chain reactions (PCR) (Skyberg et al. 2003; Ewers et al. 2005)

1.3.4 – Secreted Factors and Toxins

Bacteria secrete substances into their environment for a variety of purposes; to break down substances to uptake and metabolise, to harm other microorganisms to reduce competition for resources, to communicate with other bacteria, to improve their ability to colonise a host or to enable movement into a different niche. Some secreted factors exit the cell via secretion systems, of which there are seven types, which are briefly summarised in the following table.

Type	Overview	Example
I	ABC transporters, independent from the Sec pathway, able to transport proteins from the cytoplasm to the outer membrane without a periplasmic intermediate (Delepelaire 2004; Tseng et al. 2009).	Haemolysin *E. coli*
II	Complex assembly of 12-16 accessory proteins closely related to type 4 pili, involved in secretion of extracellular enzymes and toxins. Requires Sec/Tat pathways for transport across inner membrane (Cianciotto 2005; Tseng et al. 2009).	Enterotoxin *E. coli*
III	Sec independent pathway, assembly of 20 different components, sometimes with a periplasmic intermediate, able to inject effector proteins such as virulence factors directly into host cytosol *via* a hollow needle similar to the basal body of flagella (Cornelis 2006; Tseng et al. 2009).	*S.* typhimurium SPI-1, SPI-2.
IV	Can transport nucleic acids and proteins directly into plant, animal, yeast and bacterial cells. Spans inner and outer membranes (Tseng et al. 2009; Christie et al. 2014).	CagA antigen *Helicobacter pylori*
V	3 subclasses. Requires Sec pathway for transport into periplasm. Often important for virulence in plants and animals (Henderson et al. 2004; Tseng et al. 2009).	AIDA-1 *E. coli*
VI	Most recently characterised, similar to phage tail, can transport effector proteins directly into host cytoplasm (Bingle et al. 2008; Tseng et al. 2009).	*Vgr* locus *V. cholerae*
VII	Found in Gram positive bacteria with mycomembranes such as Mycobacteria (Abdallah et al. 2007; Tseng et al. 2009).	ESX-1, ESX-5 *Mycobacterium bovis*

Table 1: Overview of Bacterial Secretion Systems

1.3.4.1 – Vacuolating Autotransporter Toxin

The vacuolating autotransporter toxin, encoded for by *vat*, was first discovered in an APEC isolate (Parreira and Gyles 2003) after it was observed that some APEC isolates produced a vacuolating cytotoxin similar to the VacA toxin in *Helicobacter pylori* (Salvadori et al. 2001). Vat is an extracellular protease, secreted by a Type Va Secretion System, however so far there have been no substrates identified upon which Vat has proteolytic activity (Dautin 2010).

Deletion mutants were avirulent in both respiratory and cellulitis models of disease (Parreira and Gyles 2003) and its prevalence in APEC populations is often around or over 50% (Ewers et al. 2004; Oh et al. 2011; Pires-dos-Santos et al. 2013) however it is sometimes lower (Kemmett et al. 2014). It is also seen in UPEC isolates (Restieri et al. 2007; Ewers et al. 2007).

1.3.4.2 – Haemolysins

Haemolysins are an important virulence factor of extra-intestinal *E. coli*. In UPEC and NMEC strains, *hlyA* is secreted by a type 1 secretion system encoded for by *hlyABCD* and *tolC* (Welch et al. 1992) In APEC strains, *hlyF,* a putative homologue to the 'silent' K12 haemolysin *sheA* has been identified (Reingold et al. 1999; Morales et al. 2004).

The alpha haemolysin (*hlyA*) and its accompanying secretion system has been well characterized, it is a 100kDa secreted protein that forms pores and is able to lyse a variety of cell types (Welch et al. 1992).

In contrast, *hlyF* is less well characterized. Whilst it is heavily linked with virulence in APEC isolates and used as a marker for pathogenicity in virulotyping PCR panels (Johnson et al. 2008b; Van der Westhuizen and Bragg 2012; Dissanayake et al. 2014), only recently has a potential role for HlyF been identified. Murase et al. observed that HlyF promoted the production of outer

membrane vesicle, which was subsequently associated with the release of toxins. A *ΔhlyF* mutant of χ7122 was also observed to be less virulent in a chicken systemic infection model, providing further evidence of its role in pathogenicity (Murase et al. 2016).

hlyF is located in a conserved region on ColV and ColBM plasmids, along with virulence genes such as *iss*, aerobactin, salmochelin and the *sitABCD* locus (Johnson and Nolan 2009) and it has been shown to be present in low number of commensal isolates (Dissanayake et al. 2014).

1.3.4.3 – *ibeA*; invasion

ibeA encodes for a 50kDa protein, which has been implicated for virulence in both APEC and NMEC infections. Whilst the exact function is still unknown, deletion mutants have reduced virulence in rat meningitis models (Huang et al. 1995) and chick challenge models (Germon et al. 2005), additionally deletion mutants regained virulence when complemented with a plasmid containing *ibeA* (Wang et al. 2011).

In NMEC infections, it is thought that IbeA plays a role in **i**nvasion of **b**rain microvascular **e**ndothelial cell invasion, allowing the bacteria to translocate across the blood brain barrier (Huang et al. 1995). Another study demonstrated that along with reduced virulence in an avian model, deletion mutants were significantly less able to produce biofilms and less able to invade, colonise, and proliferate in tissues such as the lung, brain (Wang et al. 2011), liver, and blood (Germon et al. 2005) which suggests a role for *ibeA* in migrating from the lungs to cause a more systemic infection. Deletion mutants have also been shown to have down-regulation of other virulence genes involved in adhesion and invasion of host tissues, which would also have a negative effect on colonisation and proliferation within the host (Cortes et al. 2008; Dai et al. 2010; Wang et al. 2011).

Furthermore, recent work has suggested an additional role for IbeA in increasing resistance to H_2O_2, however this resistance does not protect against the oxidative burst within macrophages and heterophils, as they observed no difference in intracellular macrophage survival between the mutant and wild-type strain. They suggested that IbeA could be involved in protecting the bacteria from the highly oxygenated environment of the lungs earlier on in the infection process (Fléchard et al. 2012).

1.3.4.4 – Cryptic Type 3 Secretion System

Escherichia coli is known to have a cryptic Type III Secretion System known as ETT2. The gene cluster for this secretion system is known to contain homologues to the *Salmonella* pathogenicity islands Spi-1, Spi-2 and Spi-3. Possession of the gene cluster is high amongst studied strains, however the gene cluster has been shown to have undergone significant mutations and is not capable of expressing a functional T3SS (Ren et al. 2004).

However, whilst many ETT2 gene clusters are degenerate, this does not mean that ETT2 does not play a role in pathogenesis. A study by Ideses et al showed that the presence of this gene cluster in a septicaemic APEC isolate contributed to virulence. The creation of a null mutant lacking genes coding for the putative inner membrane ring resulted in a significant decrease in virulence in a 1-day-old chick model of infection, normal virulence was restored with the addition of a plasmid containing the deleted genes (Ideses et al. 2005)

1.4 – Genetics of *Escherichia coli*

Escherichia coli as a species is incredibly diverse and versatile. It encompasses both pathogens and commensals, of which the pathogens themselves are able to cause an array of dissimilar illnesses. To do this, the genome of *E. coli* has a high degree of plasticity. Of the many fully-sequenced *E. coli* isolates, it has been observed that they share a core genomic backbone of around 2000 genes and an accessory genome (also known as a pan-genome) of over 18,000 genes (Touchon et al. 2009), as illustrated below in Figure 3. This pan-genome allows for a large degree of flexibility and adaptation *via* horizontal gene transfer.

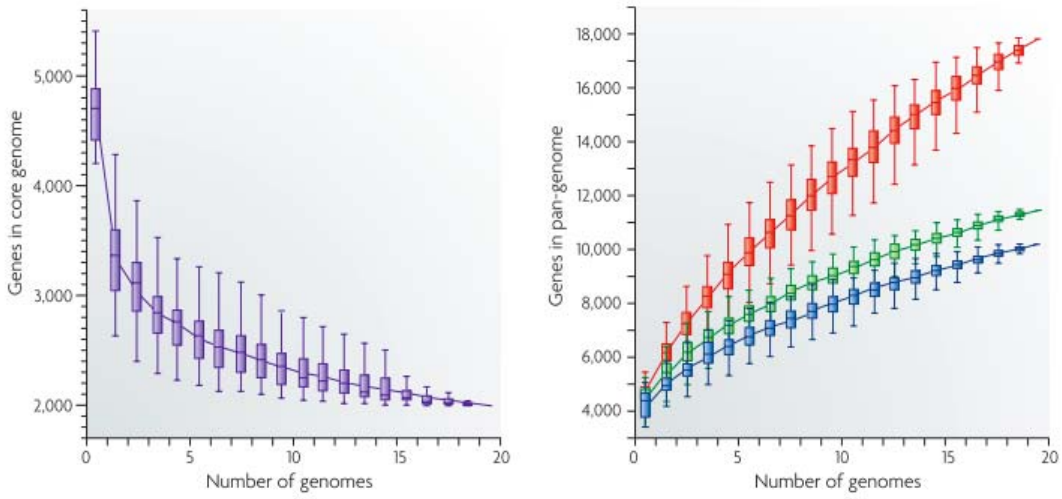

Figure 3: Genes in the *E. coli* core genome and pan-genome

Red bars indicate total genes. Green bars indicate genes with over 80% sequence homology. Blue bars indicate total genes when insertion sequences and phage regions are discounted. (Tenaillon et al. 2010)

Despite this high degree of plasticity, the core genome remains relatively conserved, with few rearrangements (Tenaillon et al. 2010), and horizontal gene transfer occurring in particular hotspots within the genome, such as transfer RNA (tRNA) regions or phage integration sites (Tenaillon et al. 2010). Horizontal gene transfer allows for both loss and gain of genetic material, which keeps the average *E. coli* genome around 4-5Mb in size (Rasko et al. 2008; Touchon et al. 2009).

1.4.1 – Next-Generation Sequencing, a seismic shift for genomics

Whilst the advent of genome sequencing is thought to be one of the most important developments in biological sciences of the twentieth century, the monumental improvements resulting in Next Generation Sequencing (NGS) technology could turn out to be one of the most important developments of the twenty-first century. The dramatic decrease in cost and increase in accuracy and speed has opened up new avenues of research and we are still only at the beginning of understanding its potential.

NGS has impacted many fields of microbiological research and is beginning to have a role in clinical practise and diagnostics. Because NGS methods are quicker and cheaper (and becoming ever more so over time) than previous sequencing methods such as Sanger sequencing, and there have been comparable advances in the analytical softwares, entirely new avenues of potential have opened such as metagenomics, the study of microbial communities. The revelations so far concerning the human microbiome and it's many roles in health and disease, such as the involvement of the gut flora in an array of conditions including, but not exclusive to: allergies, autism, Celiac disease, Crohn's and non-Crohn's inflammatory bowel diseases, gastric cancer, obesity, eating disorders, Type 2 diabetes and prognosis post-allogeneic haematopoietic stem cell transplants (Clemente et al. 2012). It's also thought that a link exists between the brain and the neurological system, termed the gut-brain axis, and although we are only at the beginning in terms of our understanding, this looks like a promising area in terms of enhancing our knowledge of mental health disorders in that they may have a microbial component that can be influenced (Cryan and Dinan 2012).

NGS has also impacted epidemiological studies. It is now possible to temporally and geographically monitor fluctuations in bacteria populations (Baker et al. 2010). It also enables public health organisations to react promptly to new outbreaks, such as the 2010 Cholera outbreak in Haiti, (Chin et al. 2011) or the

E. coli O104:H4 outbreak in Germany which was identified after isolates underwent NGS and the information released online to undergo crowd source investigation by scientists worldwide (Rohde et al. 2011; Mellmann et al. 2011).

Improvements in next-generation sequencing technology has also resulted in a huge increase in the number of sequenced *E. coli* isolates since the publication of the first *E. coli* genome in 1997 (Blattner et al. 1997). As of July 2016 there are 97 fully annotated gapless chromosome sequences in Genbank for *E. coli* and over 800 scaffold assemblies.

These sequenced genomes have provided a wealth of information on the genetics of *E. coli,* which, as a model organism, allows us to extrapolate to other related bacteria but also, as an important pathogen, allows us to explore new avenues of research for monitoring and control of *E. coli*.

1.4.2 – Phylogenetics

Phylogenetic techniques allow us to study the evolution and population structure of groups of organisms by clustering them into groups based on similarities within their genomes. It also allows us to evaluate evolutionary change over time, examine conservation of genes, and estimate the time frame over which a bacterial ancestor evolved into closely related but separate bacterial species such as *E. coli, Yersinia pestis, Salmonella enterica and Klebsiella pneumoniae*, which all diverged from a common *Enterobacteriaceae* ancestor (Baumler et al. 2013).

E. coli are phylogenetically clustered into four groups: A, B1, B2 and D, of which ExPEC usually belong to groups B2 and D (Selander and Levin 1980; Selander et al. 1987; Herzer et al. 1990) however extensive recombination within the species obscures the earliest divergences (Johnson et al. 2006a).

It has also been suggested that phylogenetic techniques could be less valid for ExPEC clonal groups. In one study, 9 ExPEC clonal groups were shown to be considerably homogeneic with regards to genomic sequence despite representing faecal isolates and a range of geographically diverse pathogenic isolates from a range of host species causing diverse clinical syndromes. The authors posit that within the ExPEC B2 group, predictions of clinical syndrome, geographical location, host species and pathogenicity are unfounded due to the sheer variety of pathogenically flexible organisms within this group and that pathogenicity at a given anatomical site is possible for organisms with a wide variety of virulence profiles and phylogenetic backgrounds (Johnson et al. 2006a).

A study on APEC and human ExPEC isolates has also observed high levels of homogeneity within these isolates, suggesting that some APEC strains are have high zoonotic potential and adding further weight to the theory that ExPEC isolates are highly similar regardless of host species and syndrome (Moulin-Schouleur et al. 2007).

1.4.3 – MLST types

Multilocus sequence typing (MLST) was first proposed as a typing method for characterising pathogenic microorganisms. By sequencing short fragments of ~6 genes under strong conserving selection pressure it is possible to group isolates into clonal clusters, and it was shown that the data generated were comparable to those generated using the previous typing method of multilocus enzyme electrophoresis (MLEE) (Maiden et al. 1998).

For typing *E. coli* the MLST protocol uses fragments of seven housekeeping genes: *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase), *recA* (ATP/GTP binding motif) to

classify *E. coli* isolates into 600 sequence types (ST) and 54 ST complexes (Wirth et al. 2006).

Whilst MLST is useful for typing bacteria, the high degree of horizontal transfer within *E. coli* populations complicates our understanding of evolution within this species. There is conflicting evidence for MLST's ability to classify pathogenic variants of *E. coli* into ST groups, using one MLST protocol appears to result in clusters that have some degree of organisation according to pathotype (Escobar-Páramo et al. 2004) however another protocol shows no grouping of pathotypes to particular clusters (Wirth et al. 2006; Turner et al. 2006). Studies using MLST data are thus limited in their accuracy with regards to evolutionary families of *E. coli*.

1.4.4 – Virulence Plasmids and Pathogenicity Islands

A key aspect of the highly variable nature of *E. coli* pathogenicity is virulence plasmids and pathogenicity islands. As a species, *E. coli* possesses a large array of plasmids including those associated with virulence. Most *E. coli* virulence plasmids fall into the IncF compatibility group (Johnson and Nolan 2009).

A group of plasmids that are particularly associated with ExPEC are ColV plasmids and their descendants ColBM plasmids (Smith and Huggins 1976). It was first observed that carriage of these plasmids was associated with iron acquisition and serum resistance (Binns et al. 1979, 1982) and sequencing of ColV and ColBM plasmids has revealed a conserved region containing virulence genes associated with ExPEC, including *iss* and *traT* involved in serum resistance (Johnson et al. 2002), and *tsh* (Provence and Curtiss III 1994). Furthermore it has been observed that an additional iron acquisition system is found on ColV plasmids (Dozois et al. 2003).

Col plasmids have been associated with a variety of functions that enable bacteria to better survive in their environments and become more successful

pathogens. These include resistance to chlorine and disinfectants, phage resistance, tolerance of acidic pH, growth in urine, and ability to cause disease in avian species, meningitis in new-borns, and infections of the human urinary tract (Johnson and Nolan 2009).

1.4.5 – Pathogenicity and Virulotyping

It was first suggested in 1993 that the majority of *E. coli* isolates causing colibacillosis fall into just a few clonal complexes (White et al. 1993). In the following years, many studies focused on trying to elucidate what causes some *E. coli* to be pathogenic and some commensal. These studies have often shown correlations between possession of certain virulence genes and pathogenicity.

Thus, it follows, if these genes are strongly linked with virulence, that we could potentially use possession of these genes as markers for pathogenicity. Indeed, a population study of 150 isolates using PCR to detect 17 different virulence genes later provided further evidence to support White's theory that the majority of APEC isolates (Janßen et al. 2001) fall into only a few clonal complexes. If this were indeed the case, then using a panel of these virulence genes in a PCR assay would be a useful and accurate diagnostic test for APEC. Soon after, two different PCR panels were suggested by APEC researchers (Skyberg et al. 2003; Ewers et al. 2005) for use as diagnostic tools alongside existing protocols. Skyberg suggested a quadraplex PCR panel to detect the genes *iss*, *tsh*, *cvi* and *iucC*. Ewers suggested an octoplex panel to detect *papC*, *iucD*, *irp2*, *tsh*, *vat astA*, *iss* and *cva/cvi*. In their respective studies these panels were able to distinguish between APEC, avian faecal *E. coli* (AFEC) and the closely related UPEC isolates also, indicating that these tests could be of great use, however these studies only had sample sizes of 20 and 40 respectively (Skyberg et al. 2003; Ewers et al. 2005). Later, a pentaplex PCR panel was suggested (Johnson et al. 2008b), which was able to clearly distinguish between a panel of nearly 1000 APEC and AFEC isolates which validated the use of PCR assays to differentiate between APEC and AFEC isolates, which they suggested

could be utilised to detect potential pathogens in the poultry house environment, along the food chain, and also in human diseases (Johnson et al. 2008b).

A further potential use for PCR virulence gene assays is characterising isolates into groups according to syndrome. A study of 79 avian *E. coli* samples showed that using virulence gene profile, phylogenetic group, adhesion to eukaryotic cells *in vitro* and LD50 assay, they were able to classify isolates into four groups representing those causing septicaemia, swollen-head syndrome, omphalitis and commensal faecal isolates (Maturana et al. 2011), however reproductive tract infections were not included in this study, and given the importance of this syndrome in the laying industry this is a large oversight.

However, there are limitations to using possession of virulence genes to differentiate between commensal or faecal isolates, and pathogenic isolates of various syndrome-causing types. High genetic diversity has been observed amongst disease-causing isolates from broilers within a flock (Kemmett et al. 2013) suggesting that even within the environment of a single hen-house, disease causing isolates are not restricted to a small number of clonal complexes. As isolates closely resembling ExPEC isolates have been observed contaminating retail poultry meat, we can assume that apparently healthy birds still harbour potentially pathogenic isolates within their gut flora (Russo and Johnson 2003; Johnson et al. 2005a, b, 2009), which has been independently confirmed (McPeake et al. 2005; Ewers et al. 2009).

Additionally, a study of O2 and O78 isolates causing septicaemia in both avian and human hosts showed that clonal division of these particular isolates was independent of host species (Ron 2006), which casts doubt on our ability to confidently differentiate between different ExPEC subtypes. Finally, it has also been suggested that rather than APEC being a well armoured pathotype involving just a few restricted and highly related clonal complexes, that in fact there is not a single APEC pathotype as such, but that pathogenicity is more of

an interaction between an opportunistic *E. coli* and increased host susceptibility caused by stress, poor welfare or existing poor health of the host (Collingwood et al. 2014).

1.5 - Aims

Despite the economic importance of the laying industry, research in the past has mainly focused on the importance of APEC infections for broilers. Laying hens are also at risk of APEC infections, though the presentation differs due to many factors including the ability of *E. coli* bacteria to ascend the oviduct when the hen is in lay (Barnes et al. 2008). When this project commenced, only one published APEC genome existed; APEC O1. This isolate was isolated from the lung of a chicken suffering from colisepticaemia, and was chosen by the Nolan group as representative of a APEC pathotype, and because it belongs to an O serogroup implicated as being important for both animal and human extra-intestinal infections (Johnson et al. 2007).

A previous study by this research group studied 70 APEC isolates recovered from an infection outbreak in a layer-breeder flock (Jordan et al. 2005), and molecular epidemiological techniques were used to examine the phylogeny and virulence profiles of these isolates. These techniques revealed that high proportion of these isolates were associated with a single epidemic clonal group that was exclusively associated with the reproductive tract. The isolate possessed both conventional APEC virulence factors and those associated with UPEC infections. This isolate was also found in the environment, suggesting it was present in the hen house prior to infection (Timothy et al. 2008).

As it has previously been shown that APEC and UPEC are exceedingly closely related, sometimes indistinguishable, this project aimed to characterize the SPS-associated isolate, using both phenotypic and genotypic techniques to determine if SPS-associated APEC isolates were a separate pathotype from colibacillosis-associated isolates, and if this SPS-associated isolate was able to so efficiently colonise the reproductive tract due to increased similarity to UPEC isolates. This will provide valuable insight for a still under-studied aspect of APEC infections, and could have implications both for the poultry industry and for human public health.

2 – General Materials and Methods

2.1 – Bacterial Strains, storage and growth conditions

E. coli strains used as the panel in this study are as follows.

E. coli 3770, the focus of this study, is an isolate associated with salpingitis in laying hens originating from an outbreak in an egg production facility in Wirral, UK.

APEC O1 and χ7122 were used to compare 3770 with other APEC strains and UTI89 was used as an ExPEC comparison. APEC χ 7122 was kindly donated by Professor Mark Stevens at the University of Edinburgh Roslin Institute, Scotland, UK. APEC χ7122 is a reference strain for the O78 pathotype and is a spontaneous mutant of the ECI strain originally isolated from the liver of a diseased turkey. A K12 isolate, MG1655 was used as a non-ExPEC comparator.

For the SPS study, *E. coli* isolates originating from outbreaks of salpingitis in commercial layer flocks were kindly donated by Ridgeway Biologicals (Compton, UK).

Samples were stored long-term at -80°C in Microbank vials (Pro-Lab Diagnostics, UK) and resuscitated using aseptic technique to streak frozen stocks onto LB agar and incubating overnight at 37°C. Stationary phase cultures were prepared by inoculating 10ml standard Miller formulation Luria-Bertani (LB) broth (Oxoid, UK) with a few colonies from a nutrient agar plate using a sterile loop. Cultures were incubated overnight for 16-18 hrs at 37°C, 150rpm in an orbital shaker. Late-logarithmic phase cultures were achieved by diluting stationary phase cultures 1:100 (v/v) into fresh LB broth followed by incubation at 37°C, 150rpm for a further 3.5 hrs.

2.2 – DNA Extractions

2.2.1 – Simple DNA Extraction for PCR

Bacteria were cultured on streak plates using LB agar, and incubated overnight at 37°C and 150rpm. One colony of bacteria was picked using a sterile loop and was added to 500µl sterile distilled water and vortexed thoroughly to suspend bacteria.

Samples were boiled for 10 mins at 95°C, then centrifuged for 10 mins at 16,000g. The resulting supernatant was removed and stored at -20°C.

2.2.2 – Extraction of High Quality DNA for Genome Sequencing

High quality genomic DNA was extracted for genome sequencing of isolate 3770 using the Promega Wizard Genomic DNA Purification kit as per manufacturers instructions with modifications as follows:

1. All centrifugation steps were altered to 5 mins at 16,000g
2. Initial bacterial cultures were layered to generate larger pellets whereby after centrifugation of bacterial cultures, the supernatant was discarded and another 1.5ml of culture added and centrifuged.
3. Step 9 incubation was 60 mins, followed by immediate chilling on ice for 5 mins.
4. Step 10, the protein precipitation solution was kept on ice, and double the amount stated was used (400µl)
5. DNA was rehydrated at 65°C for 1 hr.

2.2.3 – Quality check of extracted DNA

The Qubit dsDNA BR Assay (Thermo Scientific, UK) was used as per manufacturers instructions.

2.3 – Genome sequencing of 3770

The 3770 genome was sequenced using the HiSeq Illumina platform (San Diego, CA, USA) and assembled at the Centre of Genomic Research, University of Liverpool, using a comparison with χ7122 to order the contigs and prepare a scaffold, which then underwent annotation using Prokka (http://www.vicbioinformatics.com/software.prokka.shtml) with a custom database of all available annotated *E. coli* and *Shigella* genomes.

2.3.1 – Manual curation of 3770 genome

The 3770 genome was manually curated in order to confirm the results generated by the Prokka tool. Individual coding sequences were sent through a BLAST search using the Artemis viewer BLAST search function. These individual searches were contrasted against the predictions generated by Prokka and for sequences where the BLAST search resulted in more accurate information (such as a predicted gene with 99-100% sequence homology contrasted against a 'hypothetical protein' predicted by Prokka) the sequence annotation was updated to reflect this more accurate information.

2.4 – Multi Locus Sequence Typing

MLST was performed on the fully sequenced genome by searching for the forwards and reverse primer sequences listed on mlst.ucc.ie within the genome. Upon finding both the forwards and reverse sequences, the region in between was saved as text. Once all gene sequences had been obtained, the nucleotide sequences were entered into the MLST allele/strain query tool on mlst.ucc.ie to generate a sequence type.

2.5 – Phylogenetic typing

A whole-genome phylogenetic tree was created using Mugsy (http://mugsy.sourceforge.net/) to align the genomes of a custom database of *E. coli* and *Shigella* isolates. A neighbour-joining algorithm was applied to the aligned sequences using ClustalW (http://www.clustal.org/) to generate a phylogenetic tree.

2.6 – Population Study of SPS Isolates

Multiple PCR assays were used to study the levels of various virulence factors within this specific subpopulation of *E. coli*. These virulence factors were chosen to reflect the variety of virulence factors present in both APEC and UPEC populations.

2.6.1 – PCR Reactions

Primer sequences are shown in Table 2 and were obtained from Eurofins MWG (Germany). Each 25µl reaction mixture contained 1µl DNA, 1µl (100 pmol) each of the forward and reverse primers and 22µl of 1.1x ReddyMix™ PCR Master Mix containing 1.5 mM MgCl2 (Thermo Scientific, UK).

PCR thermocycler conditions were as follows: 94°C for 5 mins, followed by 30 cycles of 94°C for 30 seconds, Ta (see Table 2) for 1 min and 72°C for 50 seconds/500kilobases (kb) of fragment, 72°C for 7 mins, then hold at 4°C.

Primer	Sequence	Ta (°C)	Product (bp)	Reference
tsh F	GGGAAATGACCTGAATGCTGG	55	420	(Van der Westhuizen and Bragg 2012)
tsh R	CCGCTCATCAGTCAGTACCAC			
cva/cvi F	CACACACAAACGGGAGCTGTT	52	672	(Van der Westhuizen and Bragg 2012)
cva/cvi R	CTTCCGCAGCATAGTTCCAT			
ibeA F	TGAACGTTTCGGTTGTTTTG	48	814	(Germon et al. 2005)
ibeA R	TGTTCAAATCCTGGCTGGAA			
iss F	CAGCAACCCGAACCACTTGATG	57	323	(Van der Westhuizen and Bragg 2012)
iss R	AGCATTGCCAGAGCGGCAGAA			
iucC F	CGCCGTGGCTGGGGTAAG	58	541	(Skyberg et al. 2003)
iucC R	CAGCCGGTTCACCAAGTATCACTG			
iutA F	ATGAGCATATCTCCGGACG	51	587	(Moulin-Schouleur et al. 2006)
iutA R	CAGGTCGAAGAACATCTGG			
iroN F	AAGTCAAAGCAGGGGTTGCCCG	57	667	(Van der Westhuizen and Bragg 2012)
iroN R	GATCGCCGACATTAAGACGCAG			
yjjQ F	AATGGTTGTCAGCACTATGGC	53	1693	(Van der Westhuizen and Bragg 2012)
yjjQ R	GTTCAGTCAGGCAGGATAATCC			
hlyF F	TCGTTTAGGGTGCTTACCTTCAAC	52	444	(Moulin-Schouleur et al. 2007)
hlyF R	TTTGGCGGTTTAGGCATTCC			
sfaS F	CTCCGGAGAACTGGGTGCATCTTAC	58	410	(Johnson and Stell 2000)
sfaS R	CGGAGGAGTAATTACAAACCTGGC A			
cnfI F	AAGATGGAGTTTCCTATGCAGGAG	56	498	(Yamamoto et al. 1997)
cnfI R	CATTCAGAGTCCTGCCCTCATTATT			
iha F	CTGGCGGAGGCTCTGAGATCA	58	827	(Johnson et al. 2002)
iha R	TCCTTAAGCTCCCGCGGCTGA			
papA F	ATGGCAGTGGTGTCTTTTGGTG	55	717	(Johnson and Stell 2000)
papA R	CGTCCCACCATACGTGCTCTTC			
usp F	ACATTCACGGCAAGCCTCAG	55	440	(Bauer et al. 2002)
usp R	AGCGAGTTCCTGGTGAAAGC			
chuA F	GACGAACCAACGGTCAGGAT	55	279	(Clermont et al. 2000)
chuA R	TGCCGCCAGTACCAAAGACA			
hlyD F	CTCCGGTACGTGAAAAGGAC	55	904	(Johnson and Stell 2000)
hlyD R	GCCCTGATTACTGAAGCCTG			
irp2 F	AAGGATTCGCTGTTACCGGAC	51	413	(Dozois et al. 1992; Janßen et al. 2001)
irp2 R	AACTCCTGATACAGGTGGC			
kpsMTK1 F	TAGCAAACGTTCTATTGGTGC	51	153	(Johnson and Stell 2000)
kpsMTK1 R	CATCCAGACGATAAGCATGAGCA			

Table 2: Gene target, primer sequences, annealing temperature used (Ta), and product length.

2.6.2 – Visualisation of PCR Products

PCR products were run by electrophoresis on an agarose gel. Stock (10x) Tris-acetate-EDTA (TAE) buffer was prepared by dissolving 48.5g Tris base, 11.4ml glacial acetic acid and 20ml 0.5M EDTA in 1 litre dH2O. Dilution of 10x TAE buffer 1:10 (v/v) in dH2O gave a 1x working solution. 1.5% (w/v) gels were prepared by addition of 3.8g agarose into 250ml 1x TAE buffer, which was heated until the agarose had dissolved. After the molten solution had cooled slightly, 6.2µl ethidium bromide was added and the mixture was poured into a gel cast with a multi-well comb. Once the gel had set, the comb was removed and 10µl of each PCR product was loaded into the wells. A working solution of ΦX174 HaeIII digest DNA marker (Thermo Scientific, UK) was also loaded onto the gel for estimation of PCR product amplicon size. The gel was then transferred to a gel electrophoresis tank filled with (1x) TAE buffer and run at 120V for 30-40 min. Finally, the DNA products were visualised under ultraviolet light in a transilluminator.

2.7 – Growth curves

Growth curves were carried out to characterise growth of bacterial isolates (strains 3770, UTI89, APEC O1, χ7122 and K12 at 2 different temperatures reflective of a mammalian host environment (37°C) and an avian host environment (41°C).

Overnight cultures were grown in LB media at 37°C or 41°C and 150rpm and diluted 1:100 into 30ml of fresh LB in disposable flasks and placed back in for incubation under the same conditions. Every hour, 1ml of culture was collected into a cuvette for measurement of optical density at 600nm (OD_{600}) using a Multiskan FC Microplate reader (Thermo Scientific, UK). Sterile LB broth was used to blank the instrument. Samples were serially diluted and plated out to determine viable bacterial counts by the method of Miles *et al*. (Miles and Misra 1938). Briefly, serial dilutions were made in sterile phosphate-buffered saline

(PBS) and 20µl of each dilution were plated onto LB agar. Agar plates were incubated at 37°C for 20-24 hrs, after which the number of colony-forming units per ml (CFU/ml) were calculated from dilutions that produced 5 to 60 colonies.

2.8 – Presence of K1 capsule

Overnight cultures were grown in LB media at 37°C and 150rpm and diluted 1:100 into 30ml of fresh LB in disposable flask and placed back in for incubation under the same conditions for 3 hrs. 1ml of culture was transferred to a clean eppendorf tube and centrifuged to obtain a pellet. The pellet was washed with PBS and resuspended in 10mM MgSO4. 100µl of the bacterial cell-MgSO4 suspension was mixed with 100µl phage dilution buffer (100mM NaCL, 8mM MgSO4•7H2O, 50mM Tris pH7.5, 0.01% gelatine) and incubated at room temperature for 30 mins. 3ml of soft top LB agar (3% agar) was added to the reaction mixture and this was poured onto an LB plate. Plates were incubated for 18 hrs at 37°C and assessed for presence of plaques.

2.9 - Survival in Chicken Serum

To determine the ability of the panel to survive in chicken serum, survival experiments were performed in chicken serum isolated from seventy-six-week old laying hens *via* centrifugation of venous blood. Serum was pooled from 10 birds. Control serum was inactivated by heating for 30 mins at 56°C.

Overnight cultures were grown in LB media at 37°C with shaking for 18 hrs. These cultures were then diluted into fresh LB media at a ratio of 1:10 and incubated further at 37°C with shaking until at mid-log phase (measured by an OD_{600} of between 0.2 and 0.3).

Cultures were diluted in gelatine veronal buffer (TCS Biosciences Ltd, Bucks, UK) to an OD_{600} of 0.1 and 20µl of diluted culture was taken and serially diluted in

sterile PBS to 10^{-8} and 5µl of each dilution was plated out onto LB agar and incubated overnight as the inoculum.

In sterile 1.5ml eppendorf tubes, 100µl of serum was added to 900µl of gelatine veronal buffered bacterial suspension and samples were incubated for 3 hrs at 37°C.

At time points 0, 1, 2 and 3 hrs, 20µl of sample was taken. Samples were serially diluted to 10^{-8} in sterile PBS and 3 x 5µl of each dilution plated out onto LB agar. Agar plates were incubated at 37°C for 20-24 hrs, after which the number of colony-forming units per ml (CFU/ml) were calculated from dilutions that produced 5 to 60 colonies.

2.10 – *In vitro* cell studies

2.10.1 – Cell Culture

2.10.1.1 – DF1 Chicken Foetal Fibroblast cell line

The avian foetal fibroblast DF1 cell line (Himly et al. 1998) was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, UK), at 41°C with humidity (5% CO_2). Two days prior to infection, cells were seeded into 24-well tissue culture plates at a density of $4.5x10^5$ cells per well and incubated at 41°C to give a final density of $1x\ 10^6$ cells.

2.10.1.2 – HD-11 cell line

The avian macrophage HD-11 cell line (Beug et al. 1979) was grown in RPMI-1640 media (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum, 1% GlutaMAX™ (100x concentration) (Invitrogen, UK) and 1% MEM non-essential amino acid solution (Sigma-Aldrich, UK) at 41°C with humidity (5% CO_2). Two days prior to infection, cells were seeded into 24-well tissue culture

plates at a density of $4.5x10^5$ cells per well and incubated at 41°C to give a final density of $1x\ 10^6$ cells.

2.10.2 – Bacterial Culture Preparation

All bacterial isolates were resuscitated from -80°C culture collections by streaking onto LB agar and incubating overnight at 37°C. One day prior to the invasion assays, 1 colony of each overnight culture was used to inoculate 20ml of sterile LB broth. Bacterial suspensions were incubated overnight at 37°C. On the day of the infection studies, 500µl of overnight culture was added to 20ml of fresh LB broth and incubated at 37°C for 2 hrs, after which the OD_{600} was adjusted using sterile PBS to 0.1 ± 0.05 to reduce differences in multiplicity of infection (MOI) between samples. These adjusted suspensions (MOI of ~28) were used to infect the immortalised cell lines.

2.10.3 – *In vitro* infection - invasion

The growth medium was removed from the cultured DF-1 cells and the cells washed 3 times with sterile PBS and replaced with 1ml of warmed (37°C) sterile PBS, cell monolayers were incubated for a further hour at 37°C and 5% CO_2.

100µl of bacterial samples (with adjusted OD_{600}) were added to the monolayers in triplicate. Infected monolayers were re-incubated at 37°C for 1 hr. After the initial incubation time, the supernatant was removed and replaced with 1ml of growth media containing either 100µg/ml gentamicin sulphate (Invitrogen, UK) or 150 µg/ml colistin sulphate in the case of APEC O1 (Sigma-Aldrich, UK). Cells were incubated further at 37°C for 1 hr. The antibiotic containing media was subsequently removed and cells were washed once with sterile PBS then lysed with 1ml 0.5% Triton X-100 in PBS and incubated at 37°C for 5 mins. Cell lysates were serially diluted in PBS and intracellular bacteria enumerated on LB agar. Agar plates were incubated at 37°C for 20-24 hrs, after which the number of

colony-forming units per ml (CFU/ml) were calculated from dilutions that produced 5 to 60 colonies.

2.10.4 – *In vitro* invasion, intracellular survival.

The growth medium was removed from the cultured fibroblasts and the cells washed 3 times with sterile PBS and replaced with 1ml of warmed (37C) sterile PBS, cell monolayers were incubated for a further hour at 37°C and 5% CO_2.

100µl of bacterial samples (with adjusted OD_{600}) were added to the monolayers in triplicate. Infected monolayers were re-incubated at 37°C for one hour. After the initial incubation time, the supernatant was removed and replaced with 1ml of growth media containing either 100µl gentamicin sulphate (Invitrogen, UK) or colistin in the case of APEC O1 (Invitrogen, UK) was added to each well and incubated at 37°C for 1, 2, 4, or 24 hrs. This was subsequently removed and cells were washed once with sterile PBS then lysed with 1ml 0.5% Triton X-100 in PBS and incubated at 37°C for 5 mins. Cell lysates were serially diluted in PBS and intracellular bacteria enumerated on LB agar. Agar plates were incubated at 37°C for 20-24 hrs, after which the number of colony-forming units per ml (CFU/ml) were calculated from dilutions that produced 5 to 60 colonies.

2.10.5 – *In vitro* infection - adhesion

The growth medium was removed from the cultured DF-1 cells and the cells washed three times with sterile PBS and replaced with 1ml of warmed (37°C) sterile PBS, cell monolayers were incubated for a further hour at 37°C and 5% CO_2.

100µl of bacterial samples (with adjusted OD_{600}) were added to the monolayers in triplicate, with a further triplicate set for invasion control. Infected monolayers were re-incubated at 37°C for one hr. After the initial incubation

time, the supernatant on the control cells was removed and replaced with 1ml of growth media 100µg/ml gentamicin sulphate (Invitrogen, UK) or colistin in the case of APEC O1 (Invitrogen, UK) in each well and incubated at 37°C for 1 hr. For the adhesion samples, the growth media was removed at 1 hr, and cells were washed once with sterile PBS then lysed with 1ml 0.5% Triton X-100 in PBS and incubated at 37°C for 5 mins. For the invasive samples, this step was performed after 1hr incubation with growth media + antibiotic. Cell lysates were serially diluted in PBS and intracellular bacteria enumerated on LB agar. Agar plates were incubated at 37°C for 20-24 hrs, after which the number of colony-forming units per ml (CFU/ml) were calculated from dilutions that produced 5 to 60 colonies. To calculate adhesion rates, the average count for the invasive control was removed from the average count for the adhesive samples.

2.10.6 – RT-PCR for cytokine expression in cell-based infection model

2.10.6.1 – Infection

The growth medium was removed from the cultured fibroblasts and the cells washed 3 times with sterile PBS and replaced with 1ml of warmed (37°C) sterile PBS, cell monolayers were incubated for a further hour at 37°C and 5% CO_2.

100µl of bacterial samples (with adjusted OD_{600}) were added to the monolayers in triplicate. Infected monolayers were re-incubated at 37°C for 1 hr. 3 wells per plate were left as uninfected controls. After 1 hr PBS was removed and cells were washed three times with sterile PBS. Assay plates were incubated for 1 hr at 37°C in air, after which time the medium was aspirated and 350µl Lysis Buffer RLT (Qiagen Ltd., UK) containing 1% (v/v) β- mercaptoethanol added to each well. Five minutes later the cell monolayers were disrupted by agitation with a pipette tip and the cell homogenates were collected into microcentrifuge tubes

for storage at -80°C. Assays were performed in triplicate and samples were collected from 3 independent experiments.

2.10.6.2 – RNA extraction

Total ribonucleic acid (RNA) was isolated from the cell homogenates using the Qiagen RNAeasy kit (Qiagen, UK), used as per manufacturers instructions. Purified RNA samples were stored at -80°C until required.

2.10.6.3 – qRT-PCR

RNA from sample and uninfected control DF-1 cells was subjected to one-step quantitative reverse transcription PCR (qRT-PCR) using a Rotor-Gene Probe RT-PCR kit (Qiagen Ltd., UK) on a Rotor-Gene Q (Qiagen Ltd., UK). Sequence-specific TaqMan Assay primer-probe sets for avian IL-1β, CXCLI1, CXCLI2 and 28s (Life Technologies, UK) were included in the reaction tubes with 10ng sample RNA. Reaction mixtures were set up using a QIAgility instrument, performed in triplicate and normalised to eukaryotic 18S ribosomal RNA. Each reaction tube contained 10 µl Rotor Gene MasterMix, 0.2 µl Rotor Gene reverse transcription enzyme, 1 µl TaqMan gene expression assay and 1 µl RNA. The following cycling conditions were used for amplification: 50°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The relative levels of IL-1β, CXCLI1, and CXCLI2 gene expression were quantified using the 2-ΔΔCt method. The threshold for CT values was set for each gene and the average CT value for each triplicate set of samples was calculated. CT values were first normalised to the endogenous control and then to the uninfected control group. Expression levels were represented as the fold-change in expression compared to the uninfected control.

2.11 - Chicken Infections

All experimental procedures were performed by Home Office personal licence holders under project licence 40/3652. Seventy-seven-week old Lohmann Brown laying hens were obtained from a caged egg-laying farm and kept in the experimental house for 2 weeks to acclimatise to the experimental facility. The birds were split into 4 groups, with each group housed individually in separate experimental rooms. Birds were given ad libitum access to water and a vegetable protein-based diet (SDS, Witham, UK.) and maintained at a temperature of 20°C.

Birds were infected *via* aerosol route using a vaccine spray delivery system (kindly provided by Merial) containing an inoculum of 10^7 cfu/ml. 40ml of inoculum was sprayed over each bird, and an estimated 10^5 was inhaled.

Birds were monitored for overall welfare twice daily prior to infection, then every 4-6 hours after challenge. At 42 hrs post infection birds were killed by neck dislocation prior to post mortem examination.

Birds underwent post-mortem examination within 3 hrs of death, to ensure minimal exodus of gut bacteria into the body cavity. A range of organs, liver, spleen, heart, kidney, ovules and ovarian tissue, oviduct and uterus, were removed aseptically for bacteriological analysis

Tissue were processed as follows:

1. For spleen, heart and liver, tissues were homogenised in a 1:9 ratio of organ:sterile PBS in a Seward Biomaster Stomacher (Seward, UK). These samples were decimal serial diluted to a final dilution of 10^{-8} in sterile PBS. 3 x 10µl of each dilution was plated out onto EMBA agar and incubated overnight at 37°C. Counts were taken for overnight growth, and the average count for each dilution was calculated.

2. For kidney samples, tissues were homogenised in a 1:9 ratio of organ:sterile PBS. These samples were decimal serial diluted to a final dilution of 10^{-8} in sterile PBS. 3 x 20µl of each dilution were plated onto EMBA. Agar plates were incubated at 37°C for 20-24 hrs, after which the number of colony-forming units per ml (CFU/ml) were calculated from dilutions that produced 5 to 60 colonies.
3. For reproductive tract samples, tissues were homogenised in a 1:9 ratio of organ:sterile PBS. 3 x 20µl was plated onto EMBA. Agar plates were incubated at 37°C for 20-24 hrs, the presence of *E. coli* after 24 hrs was considered positive.

3 – Characterisation of Strain 3770

3.1 – Introduction

Strain 3770 was first isolated during a large study on salpingitis, peritonitis and salpingoperitonitis (SPS) in a layer breeder flock in which nearly 14,000 hens and 1379 cockerels were monitored for signs of reproductive tract infection between 26-58 weeks of age. A total of 243 birds died during the course of this study, of which the majority were examined for signs of reproductive tract infection. It was observed that nearly half of the birds showed clinical signs of SPS complex infections, and *E. coli* was subsequently recovered from a variety of organs within these birds (Jordan et al. 2005). 3770 was later described during a epidemiological study of this outbreak (Timothy et al. 2008) in which it was determined to be epidemic clonal isolate. Initial virulotyping revealed that this isolate possessed several virulence factors more common to UPEC isolates, as well as typical APEC virulence factors. The presence of UPEC virulence factors raised the hypothesis that virulence factors involved in infection of mammalian urinary tracts could also be an advantage in infections of the avian reproductive tract.

The aims of this chapter were to further characterise strain 3770 using a combination of biological and cell-based methods, to further understand the infectious capabilities of this field-isolated strain.

3.1.1 – Growth kinetics of *Escherichia coli*

E. coli is a well-described organism with a large volume of previous work undertaken specifically to investigate the growth kinetics of this organism. The rich growth medium of LB broth is frequently used to support growth of *E. coli* as it allows for high yields of bacterial cells in a relatively short amount of time; with a cell doubling time of approximately 20 minutes.

E. coli follows the characteristic growth pattern of lag phase where gene expression and protein synthesis occur, exponential or log phase where cells rapidly multiply, followed by stationary phase when the growth medium becomes depleted. It has previously been shown that in LB broth, stationary phase begins at an OD_{600} of 0.3 (Sezonov et al. 2007).

E. coli is frequently grown at 37°C, however the body temperature of avian species is 41°C. Previous studies have shown that the growth kinetics of *E. coli* remain comparable until 43°C after which disruption in the exponential growth phase is observed due to the activation of heat shock proteins (Van Derlinden et al. 2008).

3.1.2 – Bactericidal activity of serum

Serum is what remains from blood plasma after blood cells and clotting factors are removed. Serum is both bactericidal and bacteriolytic activity, killing bacteria *via* complement and lysozyme mechanisms. Exposure of Gram-negative bacteria to serum does not kill the bacteria instantly; there is a lag period, which differs between strain and involves factors such as growth phase and overall susceptibility to serum (Taylor 1983). In particular, bacteria which express lipopolysaccharide (LPS) are less sensitive to serum than strains lacking LPS (Rowley 1968). Susceptible bacteria such as *E. coli* K12 have a lag period so short that is undetectable (Taylor 1983).

Complement is a component of the innate immune system, in which successive complement factors are deposited onto a bacterial cell surface. Different complement activation pathways can occur reducing the potential for resistance to develop. Once the complement cascade is activated on the bacterial cell surface a pore-forming membrane attack complex (MAC) is formed. The formation of pores in the cell membrane disrupts concentration gradients within the cell and allows lysozyme to enter the cell ultimately leading to rapid bacterial cell death (Miajlovic and Smith 2014).

The ability to withstand the bactericidal action of serum is clearly an important advantage for ExPEC that access or are otherwise exposed to the bloodstream of the host. Multiple factors are involved but broadly they can be categorised as factors such as capsules or LPS which provide physical protection for the bacterium against factors such as complement, secreted factors that interfere or inhibit the complement cascade (Miajlovic and Smith 2014).

Specific ExPEC genes that are thought to play a significant part in serum resistance include *traT* and *iss.* TraT is an outer membrane lipoprotein involved in serum resistance by preventing complement-mediated killing. It is thought that TraT is able to enhance serum resistance by preventing the assembly of the MAC complex (Miajlovic and Smith 2014).

iss is more frequently carried by pathogenic avian *E. coli* than by faecal avian *E. coli* and the gene is found both on the ColV/ColBM plasmids and within the *E. coli* chromosome (Johnson et al. 2008c). *iss* is thought to originally derive from lambda-phage, having a large degree of similarity to the *bor* gene which encodes a lipoprotein in the lambda phage cell envelope (Chuba et al. 1989). So far, the mechanism of action of the *iss* gene product is still unknown.

Serum resistance is a widespread trait of APEC, and it is thought to play a major role in pathogenesis (Mellata et al. 2003a; Nolan et al. 2003) as serum resistance is often a characteristic of isolates originating from APEC outbreaks in chicken and turkeys and has been associated with virulence and lethality in chick infection models (Wooley et al. 1992; Dozois et al. 1992).

3.1.3 – Bacterial-host cell interactions

During colonisation of a host a number of bacterial-host cell interactions occur. In brief, these interactions include adhering to the host cell surface, entering a non-phagocytic host cell (invasion), and being taken up by a host white blood cell (phagocytosis). These will be described in further detail below.

3.1.3.1 – Adhesion

Bacterial cells are able to express a variety of cell surface factors, known as adhesins, which enable them to secure themselves to a particular area within their environment. Adhesion is the first step in successful colonisation, thus a vital factor for any bacterial pathogen. Different adhesins have affinities for different host cell surface factors, thus bacteria often possess genes for multiple adhesins.

In ExPEC isolates, adhesins include fimbriae (type 1 fimbriae, P fimbriae, curli fibres, S fimbriae, F1c fimbriae, and Dr fimbriae), afimbrial adhesins, haemagglutinins and more yet to be discovered (Antão et al. 2009b).

APEC isolates have been shown to be able to adhere to chicken epithelial cells in avian tracheal explant models of infection (Ramirez et al. 2009) and also in primary cell culture (La Ragione et al. 2000a) and experimentally infected chickens (Pourbakhsh et al. 1997a).

3.1.3.2 – Invasion

E. coli is not a conventional invasive pathogen such as *Salmonella enterica*, however some strains of APEC have been shown to be able to invade fibroblasts in comparable amounts to the invasive *Salmonella enterica* serovar Typhimurium SL1344 strain (Matter et al. 2011), and also invade an avian tracheal explant model of infection with an invasion efficiency of 0.3% (Ramirez et al. 2009). Other studies however have shown invasion efficiencies of between 0.1% and 0.01% (La Ragione et al. 2000a) potentially indicating a high degree of variability between strains.

Furthermore, transmission electron microscopy of the air sacs of chickens inoculated with APEC *via* the aerosol route have shown bacteria not only

adhering to epithelial cells but also invading epithelial cells (Pourbakhsh et al. 1997a).

There are several virulence factors found in APEC isolates that are thought to be associated with invasion of host cells. One such virulence factor is the invasin *ibeA* which is well described in NMEC infections in which it is involved in allowing the bacteria to cross the blood-brain barrier (Huang et al. 1995) and has also been shown to be important for virulence in avian models (Germon et al. 2005; Wang et al. 2011). Flagella and fimbriae have also been shown to be important for invasion and persistence in a day-old chick infection model, in which mutants deficient in flagella, type 1 fimbriae and curli fimbriae were significantly less virulent and persistent (La Ragione et al. 2000a).

3.1.3.3 – Survival inside phagocytes

Chickens have multiple cell types that are capable of phagocytosis. These include heterophils, macrophages, dendritic cells and thrombocytes (Wu and Kaiser 2011). Avian airsacs rely on an influx of heterophils and macrophages as a first line of defence against incoming bacteria (Mellata et al. 2003b), thus any bacteria that is able to withstand the bactericidal effect of phagocytosis would have a competitive advantage against bacteria that were not resistant, and would also be able to travel to other organs *via* the bloodstream.

There are several virulence factors frequently carried by APEC isolates that confer a degree of protection against phagocytosis. These include *traT*, which prevents antibody-mediated phagocytosis, opsonisation (Moll et al. 1980), and certain K and O antigens (Dziva and Stevens 2008), as well as type 1 and P fimbriae (Mellata et al. 2003b).

3.1.3.4 – Avian inflammatory responses to infection

During the process of colonisation of a host, the host immune system will respond in an effort to remove the pathogen. Broadly, the immune responses of avian species are similar to those seen in mammals, with both adaptive and innate immune systems and the ability to generate immunological memory.

Avian species have 10 known Toll Like Receptors (TLRs) (Wigley 2013), which are an important part of the innate immune system. Several TLRs are able to recognise and bind to pathogen-associated microbial patterns (PAMPs) such as peptidoglycan, LPS, flagellin and un-methylated CpG DNA. TLRs are expressed on various immune cells, and activation occurs rapidly in response to pathogens. TLR activation triggers a signalling cascade of pro-inflammatory cytokines and chemokines (Akira et al. 2006),

There are three avian pro-inflammatory chemokines, CXCLi1, CXCLi2, and CXCLi3. CXCLi1 and CXCLi2 are orthologous to human IL-8 (also known as CXCL8) and CXCLi3 has no mammalian orthologue. The receptor for the three avian inflammatory chemokines is CXCR1. Avian species also have pro-inflammatory cytokines, namely IL-1β, IL-18, and the more recently described IL-1RN and IL-36RN (Kaiser 2012).

Once the innate immune response has been triggered, the cascade of cytokines and chemokines directs phagocytic cells such as heterophils and macrophages to the site of infection (Wigley 2013). Phagocytic cells are able to recognise a variety of TLR ligands and capture bacteria. Subsequently, internalised bacteria are exposed to a variety of antimicrobial threats including reactive oxygen and reactive nitrogen species.

3.1.2 – Chicken infection models

A number of infection models exist, reflecting a variety of potential routes of infection and a variety of possible disease syndromes.

It is hypothesised that colibacillosis (and subsequent colisepticaemia) originate with bacterial colonisation of the avian air sac (airsacculitis) after which the bacteria progress to cause systemic infection. The exact route by which the bacteria travel from the airsac to other sites is still unknown but is thought to either be *via* uptake into heterophils and macrophages or *via* direct invasion of damaged airsac or lung epithelia (Dziva and Stevens 2008).

In laying birds, an important but still understudied APEC disease syndrome is salpingitis-peritonitis-salpingoperitonitis syndrome, in which the reproductive tract of the mature bird is infected. This syndrome is the most commonly seen form of APEC infection in laying birds in the UK (Jordan et al. 2005) and it is thought to originate either *via* translocation of bacteria from the left abdominal airsac to the oviduct (Dho-Moulin and Fairbrother 1999) or *via* an ascending infection of the vent (Cumming 2001).

3.1.2.1 – Infection models: respiratory complex

A large variety of infection models exist for the respiratory complex of APEC infections. These include infection *via* the intra-tracheal route in which bacteria are deposited onto the tracheal mucosa of 5 week old chickens using a gavage needle and syringe (Antão et al. 2009a) and similarly intranasal inoculation onto the anterior nares (Smith et al. 1985).

More commonly used is the intra-airsac inoculation model by which bacteria is brought immediately into direct contact with the respiratory mucosa *via* injection into the posterior thoracic or abdominal airsacs. Whilst the intra-airsac model is not a useful model for natural infections, it is a very useful tool for

studying the virulence of various APEC strains and mutants, for assessing vaccine candidates and for studying the roles of various APEC virulence genes in infection (Dziva 2010).

The aerosol inoculation route is considered to be a good model for natural field conditions (Dziva 2010), however there is no one agreed-upon route of administration. Routes include administering aerosolised *E. coli* to birds for 40 minutes *via* a nebulizer (Peighambari SM et al. 2000), and repeated exposure to aerosolised *E. coli for* 20 minutes over multiple days (Ginns et al. 2000).

3.1.2.2 – Infection models: SPS complex

As of yet, a well-described model for SPS infections does not exist within the literature. There are however, data comparing multiple different routes by which SPS infections may occur. A study by Landman et al. compared 5 inoculation routes: intra-venous, intra-peritoneal, intra-tracheal, intra-vaginal, oral and aerosol, of which all but the oral route resulted in significantly higher rates of infection than the placebo controls (Landman et al. 2013).

Intra-uterine models have been described superficially within the literature, and the model appears to have problems with low reproducibility and low infection rates (Pors et al. 2014). A recent study described a successful intra-uterine model, however this also required prior inoculation of egg yolk into the intra-peritoneal area prior to intra-uterine inoculation of bacteria (Chaudhari and Kariyawasam 2014).

Recently a model was described in which bacteria is directly inoculated into the oviduct of 48-week old commercial layers, after which post mortem examination was performed at 48 hours post infection. This model was shown to be successful in causing infection of the oviduct, and marked differences were observed between the two strains of *E. coli* used for the procedure indicating that this model could be a useful tool for investigating the virulence

of various APEC strains and also for investigating the pathogenesis of infections within the oviduct (Pors et al. 2014).

3.1.2.3 – Infection models: primary cell culture and explants

As well as *in vivo* animal models, *ex vivo* models of infection such as explants have been used to assess the role of fimbriae in adhesion and invasion of epithelial cells (La Ragione et al. 2000a; Ramirez et al. 2009). Primary cell culture have also been used experimentally for investigating the role of APEC virulence factors in the host environment or for assessing vaccine candidates (Salvadori et al. 2001; Ramirez et al. 2009; Rahman and Eo 2012).

The advantage of these studies is that it minimises the use of animals required, thus adhering to the principles of 'Replace, Refine, Reduce' that form the integral framework of humane animal research.

3.2 – Results

3.2.1 – Bacterial growth curves

To determine the growth pattern of strain 3770 compared to other ExPEC isolates, bacterial cultures were monitored over 8 hours for changes in OD_{600}. Measurement of the optical densities of the growing cultures revealed standard sigmoid curves with easily identifiable lag, logarithmic and stationary phases of growth.

At 37°C, as shown in Figure 4 below, strains 3770, UTI89 and APEC O1 all reached stationary phase at 6 hrs with an OD_{600} of around 0.35. χ7122 grew in a similar pattern, to a slightly higher final OD_{600} of around 0.4. K12 obtained a final OD_{600} of 0.25, with a slower growth pattern.

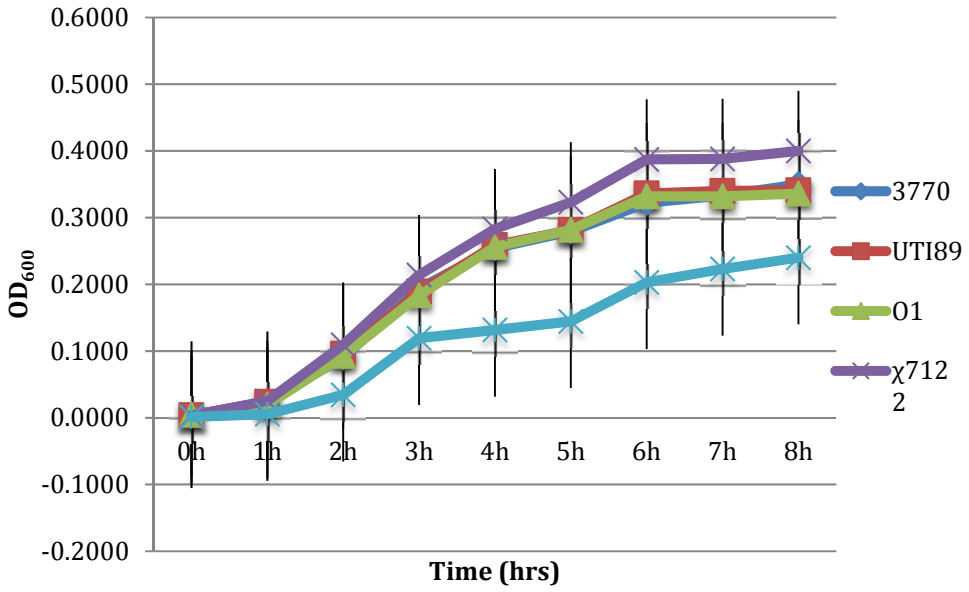

Figure 4: Growth of ExPEC isolates at 37°C.

E. coli isolates 3770, UTI89, APEC O1, χ7122 and the K12 control were grown in 30ml Luria Bertani broth in 75cm^3 polystyrene flasks at 37°C 150rpm in an orbital shaking incubator. The OD_{600} was measured every hour in a Multiskan FC plate reader against blank fresh LB broth. The experiment was performed in triplicate and results are the means of the triplicate experiments. Error bars show the standard deviation for the triplicate experiments. ANOVA testing was performed and no significant differences were observed between the isolates.

At 41°C, as shown in Figure 5 below, strain 3770 exhibited very similar growth patterns to the other isolates, reaching stationary phase at 6 hrs with an OD_{600} of around 0.5. Comparative to growth at 37°C, the strains grew slightly quicker in the early stages of the experiment and stabilized at a higher OD_{600} of 0.5.

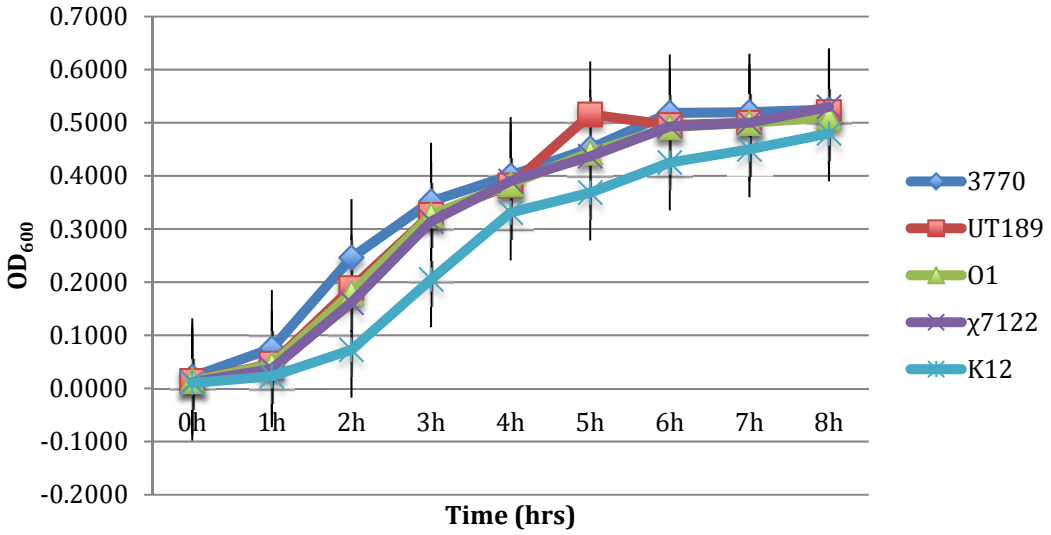

Figure 5: Growth of ExPEC isolates at 41°C.

Isolates 3770, UTI89, APEC O1, χ7122 and the K12 control were grown in Luria Bertani broth in 75cm^3 polystyrene flasks at 41°C and 150RPM. Every hour, the OD_{600} was measured in a Multiskan FC plate reader against blank fresh LB broth. The experiment was performed in triplicate and results are the means of the triplicate experiments. Error bars show the standard deviation for the triplicate experiments. ANOVA testing was performed and no significant differences were observed between the isolates.

3.2.2 – Presence of K1 phage

After 18 hrs incubation the presence of plaques in the bacterial lawn showed that the K1 capsule was present in strain 3770.

3.2.3 – ExPEC isolates were able to withstand challenge with chicken serum

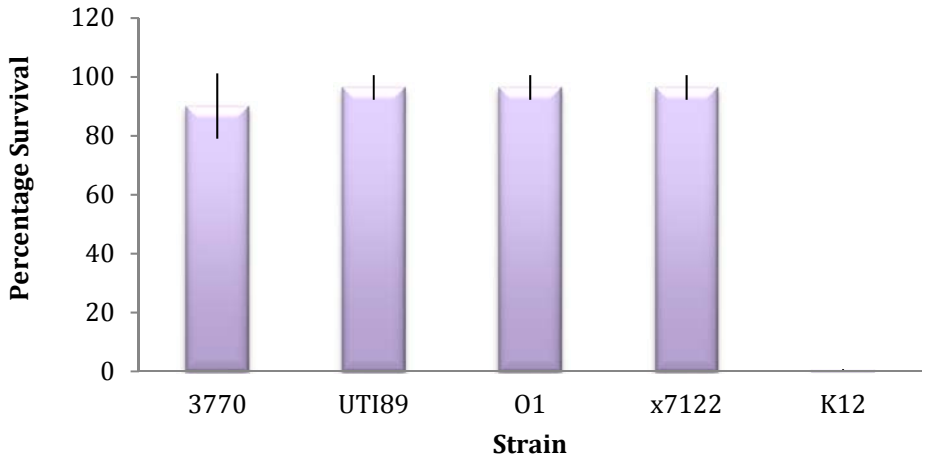

Figure 6: Survival of ExPEC isolates in chicken serum after 3 hours.

Isolates 3770, UTI89, APEC O1, χ7122 and the K12 control were grown to mid-log phase and then subsequently exposed to chicken serum for 3 hrs. Strains were also exposed to heat-inactivated serum as a control. After 3 hrs, samples were taken for numeration *via* the Miles and Misra method onto LB agar. Plates were incubated overnight at 37°C and viable counts were calculated after 18 hrs. Percentage survival was calculated against the control isolates. Experiments were performed in triplicate and results are an average of three experiments, error bars showing standard error of the mean. ANOVA testing was performed and the K12 isolate was significantly less able to grow in serum ($P<0.01$) than the other isolates.

To quantify the ability of isolate 3770 to survive in chicken serum compared to other ExPEC and the K12 control isolate, mid-log phase isolates were exposed to chicken serum for 3 hours, the results are shown in Figure 6, above. All 4 ExPEC isolates: 3770, UTI89, APEC O1 and χ7122 were able to survive with percentage survival of over 90% indicating very little if any cell death. The K12 control was unable to withstand exposure to chicken serum, with 0.42% survival after 3 hours, this difference was statistically significant ($P<0.01$).

3.2.4 – ExPEC isolates were able to adhere to avian DF-1 cells

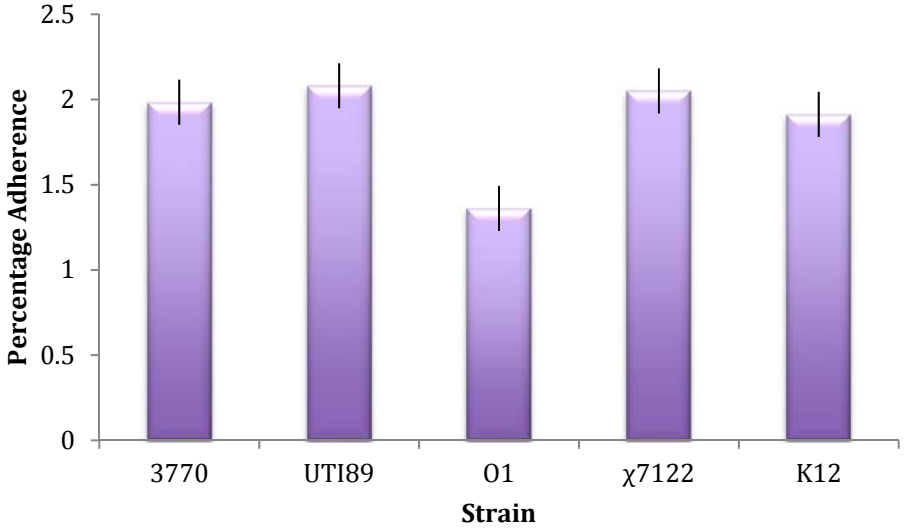

Figure 7: Adhesion to DF-1 chicken foetal fibroblast cell line by ExPEC isolates.

3770, UTI89, APEC O1, χ7122 and the K12 control were placed onto confluent monolayers of DF-1 cells and incubated for 1 hr at 41°C, after which half of the samples received antibiotic-containing growth media to kill extra-cellular bacteria and half did not receive antibiotic-containing growth media. After an additional hour, cell monolayers were washed and then lysed. Samples were taken for numeration *via* the Miles and Misra method and viable counts calculated after 18 hrs growth on LB agar at 37°C. Percentage of adhesion was calculated by removing the average count of cells that had invaded the fibroblasts from the overall total of bacterial cells within the non-antibiotic containing cells. Experiments were performed in triplicate and results are an average of three experiments, error bars showing standard error of the mean. ANOVA testing was performed and the O1 isolate was significantly less adhesive to DF-1 cells ($P<0.05$) than the other isolates.

To investigate the ability of 3770 to adhere to chicken cells, adhesion assays were performed as shown in Figure 7, above. Strains 3770, UTI89, χ7122 and K12 were equally able to adhere to DF-1 chicken fibroblasts with around 2% of the inoculum adhering to the DF-1 cells. APEC O1 was slightly less adhesive, with 1.4% of the inoculum adhering to the DF-1 cells, this difference was statistically significant ($P<0.05$).

3.2.5 – ExPEC isolates did not display an invasive phenotype

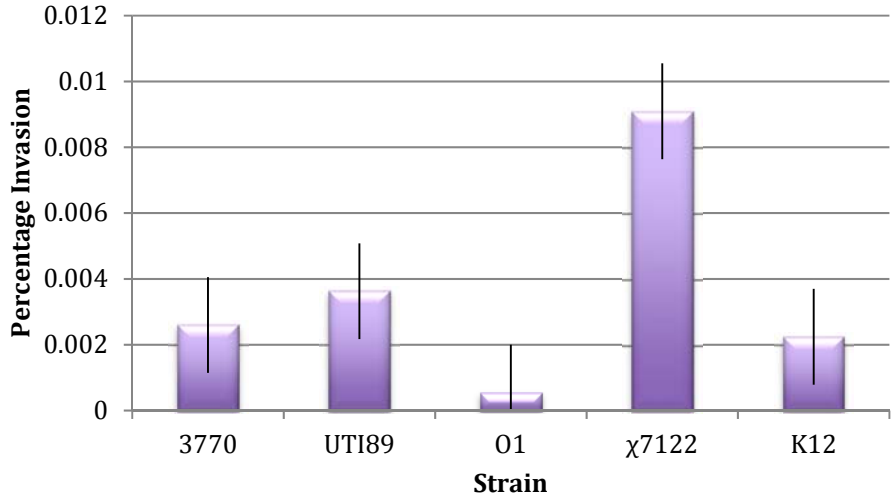

Figure 8: Invasion into DF-1 chicken foetal fibroblast cell line by ExPEC isolates.

3770, UTI89, APEC O1, χ7122 and the K12 control were placed onto confluent monolayers of DF-1 cells and incubated for 1 hr at 41°C, after which extra-cellular bacteria were killed by the addition of antibiotic-containing growth media. After an additional hour, cell monolayers were washed and then lysed. Samples were taken for numeration *via* the Miles and Misra method and viable counts calculated after 18 hrs growth on LB agar at 37°C. Experiments were performed in triplicate and results are an average of three experiments, error bars showing standard error of the mean. ANOVA testing was performed and isolate χ7122 was more capable of invading DF-1 cells ($P<0.01$) than the other isolates.

To investigate the ability of 3770 to invade chicken epithelial cells, invasion assays were performed as shown in Figure 8, above. The results varied greatly by strain. Strains 3770, UTI89 and K12 showed levels of invasion into the DF-1 cells of between 0.002-0.0035% of the inoculum. APEC O1 was less invasive than these strains, 0.0005% of the inoculum was recovered from within the DF-1 cells. χ7122 was the most invasive strain, with slightly over 0.009% of the inoculum recovered from within the DF-1 cells. This difference was statistically significant ($P<0.01$).

All strains were unable to achieve 0.01% invasion, which is the lowest observed invasion efficacy of an invasive APEC strain (La Ragione et al. 2000a).

3.2.6 – ExPEC isolates show short-term persistence in HD-11 cells

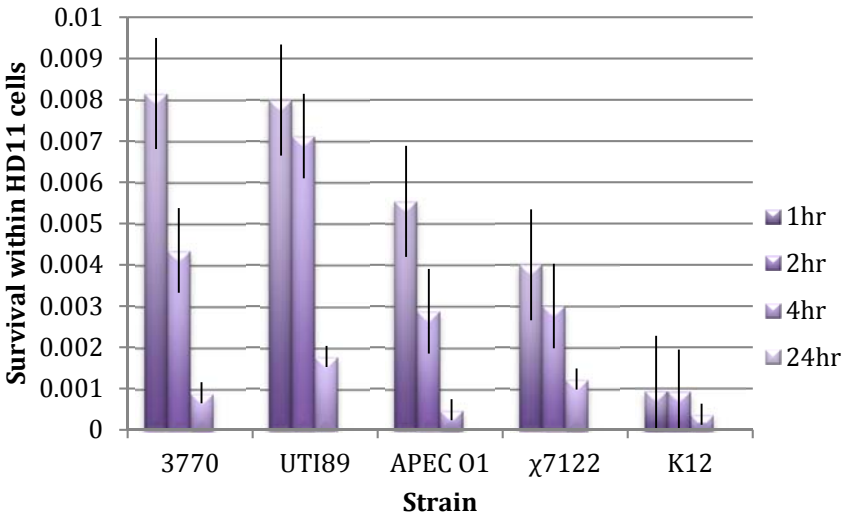

Figure 9: Persistence in HD-11 chicken macrophage cell line by ExPEC isolates.

3770, UTI89, APEC O1, χ7122 and the K12 control were placed onto confluent monolayers of HD11 cells and incubated for 1 hr at 37°C, after which extra-cellular bacteria were killed by the addition of antibiotic-containing growth media. After 1, 2, 4 or 24 hrs, cell monolayers were washed and then lysed. Samples were taken for numeration *via* the Miles and Misra method and viable counts calculated after 18 hrs growth on LB agar at 37°C. Experiments were performed in triplicate and results are an average of three experiments, error bars showing standard error of the mean. ANOVA testing was performed and after both 2hrs and 4 hrs UTI89 was statistically more persistent ($P<0.05$) than the other isolates at these time points. There was no statistical significance between the isolates at 24 hrs.

To investigate the ability of 3770 to persist within chicken macrophages, persistence assays were performed as shown in Figure 9, above. Survival is shown as a percentage of the inoculum recovered from within the HD-11 cells. For all strains, survival decreased over time. Strain 3770 decreased from 0.008% at 1 hr, to slightly above 0.004% at 2 hrs, and 0.0008% at 4 hrs. Strain UTI89 decreased from 0.008% at 1 hr, to 0.007% at 2 hrs, and 0.001% at 4 hrs. Strain APEC O1 decreased from 0.005% at 1 hr to 0.002% at 2 hrs and 0.0005% at 4 hrs. Strain χ7122 decreased from 0.004% at 1 hr to 0.003% at 2 hrs and 0.001%

at 4 hrs. Strain K12 was stable at slightly below 0.001% after 1 hr and 2 hrs, then decreased to 0.0003% after 4 hrs.

For all strains, no bacteria were recovered after 24 hrs. At 2 and 4 hrs UTI89 was statistically more persistant than the other isolates ($P<0.05$) however by 24 hrs there was no significant difference observed between the isolates.

3.2.7 – All isolates triggered a pro-inflammatory response in DF-1 cells

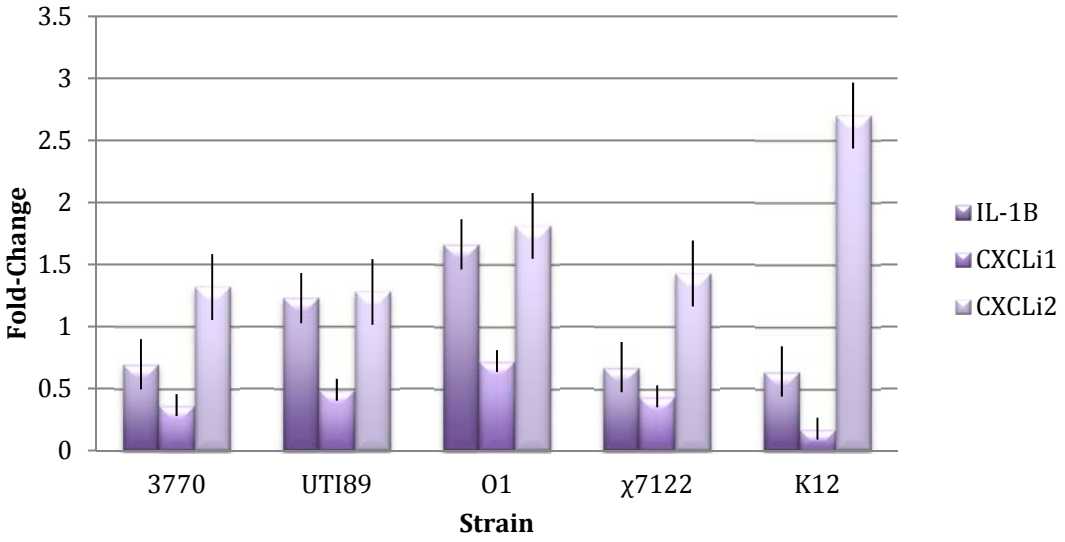

Figure 10: Expression of inflammatory markers in DF-1 cells during infection with ExPEC.

DF-1 cells were infected with 3770, UTI89, APEC O1, χ7122, and K12 as a control. RNA was extracted from washed cells from multiple experiments, and underwent RT-PCR with primers for IL-1β, CXCLi1 and CXCLi2. Values shown are the results of one experiment, error bars showing standard error of the mean. ANOVA testing was performed . For IL-1B and CXCLi1 production there was no significant difference observed between isolates. For production of CXCLi2 by isolate K12 was significantly higher ($P<0.01$) than in other isolates.

RT-PCR was performed on DF-1 cells that had been infected with bacteria to investigate the pro-inflammatory cellular responses to infection. Results are shown above in Figure 10, as a fold-change compared to non-infected cells.

For all strains, the highest seen fold-change was in CXCLi2, however the exact change varied between 1.3% and 2.7%. The next highest fold-change was seen in IL-1β, which was seen at between 0.6 and 1.66% fold change. Finally CXCLi1 was seen at between 0.18% and 1.8% fold change. The strains that triggered the lowest immune response were 3770 and χ7122. K12 triggered low fold-change in IL-1β and CXCLi1 but a high level of CXCLi2. APEC O1 triggered notable

changes in secretion of IL-1β and CXCLi2. No inflammatory response specific to ExPEC was observed. Statistical significance was only observed for production of CXCLi2 by isolate K-12. The differences between all other data points were not significant.

3.2.8 – 3770 was able to cause reproductive tract infections *via* aerosol

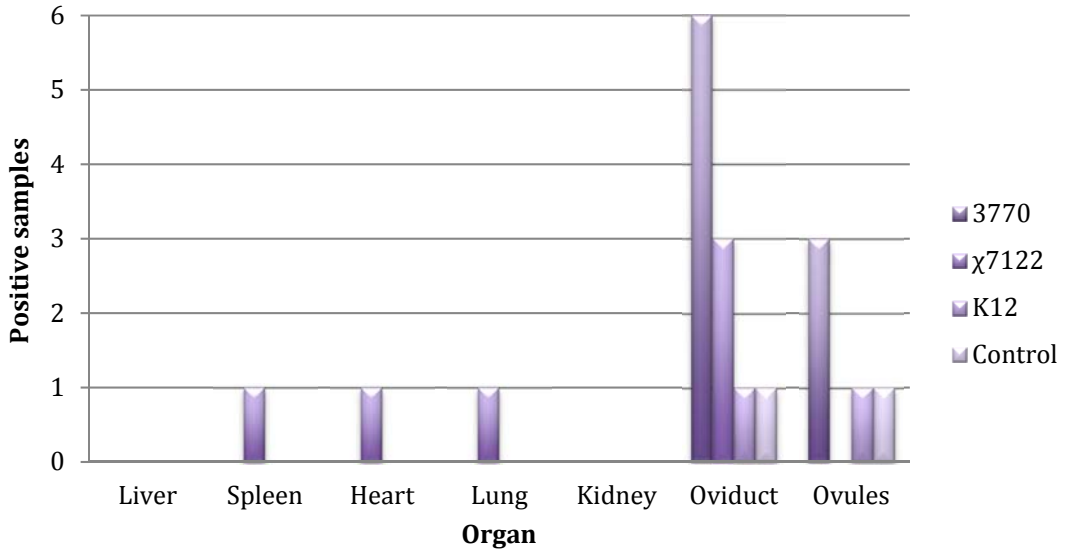

Figure 11: Number of chicken organs positive for *E. coli* 48 hours post-inhalation.

Samples were taken during post-mortem and promptly pulverised with phosphate-buffered saline solution 1:9 ratio. Samples were plated in triplicate onto EMBA agar and numerated after 18 hrs. Plates with growth after 18 hrs were counted as positive for *E. coli* within that organ. ANOVA testing was performed and isolates 3770 and χ7122 were more able to cause infection than the K12 isolate or control with significance of ($P<0.05$) and ($P<0.01$) respectively.

To quantify the ability of 3770 to cause infection *via* the inhalation route, 24 birds were infected with aerosolised bacteria and euthanized *via* cervical dislocation after 48 hrs. The results are shown above in Figure 11.

Post-mortems were carried out and the liver, spleen, heart, lung, kidney, ovules and oviducts tested for the presence of *E. coli*. Strain 3770 was more able to infect the reproductive tract of the chickens *via* the aerosol route with 6/6 birds positive in oviducts and 3/6 birds positive in the ovules. χ7122 infected the spleen, heart and lung of one bird, indicating systemic infection, and was also found in ovarian scrapings of 3/6 birds. K12 was found in the oviduct and ovules of 1 bird. The oviduct and ovules in one control bird were positive for *E. coli,*

potentially indicating a natural infection within this bird. Both 3770 and χ7122 were more able to cause infection than the K12 isolate and the control broth.

3.3 – Discussion

This chapter aimed to further characterise strain 3770 using a combination of biological and cell-based methods, to further understand the infectious capabilities of this field-isolated strain. Previously, this isolate had been isolated during a study monitoring a layer breeder flock in the North West, UK for the presence of SPS complex infections (Jordan et al. 2005) and further described in a subsequent epidemiological study of this SPS complex outbreak (Timothy et al. 2008), but beyond initial PCR-based virulotyping no further work had been done to characterise this isolate.

This chapter begins to describe the physiological characteristics of this isolate in more detail, for the first time.

3.3.1 – 3770 shows characteristic *E. coli* growth kinetics

As shown in Figures 4 and 5, all isolates exhibited characteristic growth kinetics with visible lag, exponential and stationary growth phases clearly visible. At both 37°C and 41°C all ExPEC isolates reached stationary phase after 6 hrs with a final OD_{600} of 0.35 at 37°C and 0.5 at 41°C At 37°C χ7122 grew to a slightly higher OD_{600} of 0.4 however no difference was observed at 41°C. The K12 isolate grew at a slower rate at 37°C to a final OD_{600} of 0.25 however no difference was observed at 41°C.

This data is in agreement with that seen elsewhere within the literature. Typical growth kinetics at 37°C in LB broth are as described by Sezonov et. al. in which stationary phase stops at an OD_{600} of 0.3 due to a lack of utilizable carbon sources, however growth can continue slowly up to a much higher OD_{600} of 7 (Sezonov et al. 2007). This data is also in agreement with that seen in Van Derlinden et al, in which growth kinetics of *E. coli* are unaltered from the kinetics seen at 37°C (Van Derlinden et al. 2008). The most notable difference within the two experiments in this study is a slightly higher OD_{600} seen in the

41°C data however this could be due to minor differences in the original inoculum size rather than altered growth kinetics.

The implications of these isolates being able to grow with no difference in their growth kinetics at both human or mammalian body temperature of 37°C and an avian body temperature of 41°C indicates that an altered host body temperature would not be a factor that would hinder or advantage during infection.

3.3.2 – Presence of K1 capsule as tested by phage assay

The K1 phage assay showed that the K1 capsule was expressed by strain 3770. As discussed later, the entire operon for this gene is present in strain 3770 and such this result is not unexpected.

3.3.3 – Survival in chicken serum

As shown in Figure 6, all ExPEC isolates were able to withstand the bactericidal effects of serum with little to no cell death seen. The K12 isolate was significantly ($P<0.01$) more susceptible to the effect of serum, with no cells recovered after 3 hrs. Resistance to serum has been shown to correlate with high levels of lethality in chick embryos (Nolan et al. 2003), providing further evidence that all 4 ExPEC isolates in this study are highly virulent.

As previously discussed, serum resistance is a well-characterised virulence factor of ExPEC, especially APEC (Mellata et al. 2003a; Miajlovic and Smith 2014). The ability to withstand serum provides a competitive advantage when in the host environment, and is likely to be an important factor in allowing the bacteria to progress from colonisation to infection.

The visible ExPEC serum-resistant phenotype within this data is not unexpected given that these isolates have been isolated from field outbreaks and are

therefore proven capable of causing disease. The data further supports the importance of serum-resistance in APEC pathogenicity.

3.3.4 – Bacterial-host cell interactions

Cell based-studies were carried out to characterise 3770's ability to interact with host cells. For these experiments, cultured monolayers of DF-1 chicken foetal fibroblasts and HD-11 avian macrophages were used as the host cells.

3.3.4.1 – 3770 has an adhesive phenotype

The ability of strain 3770 to adhere to avian DF-1 fibroblasts is shown in Figure 7. After 1 hr, 2% of the inoculum was adhered to the DF-1 fibroblasts. Similar results were seen for UTI89, χ7122 and K12. APEC O1 was slightly less invasive with 1.4% of the inoculum adhered to the DF-1 cells after 1 hr, which is statistically significant ($P<0.05$) This data is congruent with previous published studies, in which APEC strains, and fimbriated K12 strains were able to adhere to chicken epithelial cells (Pourbakhsh et al. 1997a; La Ragione et al. 2000a; Lymberopoulos et al. 2006; Ramirez et al. 2009).

As previously discussed, the ability to adhere to host cells is an essential step in colonisation and eventual infection of the host. Multiple virulence factors within APEC population confer an adhesive phenotype (Antão et al. 2009b), some of which will be discussed in Chapter 5.

An interesting observation from this experiment is that UTI89 is equally able to adhere to chicken epithelial cells as the APEC isolates within the study. A series of recent epidemiological studies have shown that consumption of chicken meat is linked with human urinary tract infections (Jakobsen et al. 2010, 2012; Bergeron et al. 2012). That UTI89 was able to adhere to avian epithelial cells, and APEC are able to cause infections in human hosts leads to the interesting

suggestion that ExPEC carry a variety of adhesins allowing them to equally adhere to both avian and mammalian cell surface receptors.

3.3.4.2 – 3770 did not display an invasive phenotype

As shown in Figure 8, strain 3770 did not display an invasive phenotype in this study, nor did the other ExPEC isolates UTI89, APEC O1, and χ7122. Other studies on UTI89 and χ7122 have revealed them to have invasive phenotypes (Herren et al. 2006; Eto et al. 2007; Mellata et al. 2010; Zhang et al. 2014) and previous studies using other APEC isolates have shown invasive capabilities (La Ragione et al. 2000a; Matter et al. 2011), including into DF-1 cells (Wang et al. 2011) and into tracheal epithelial cells (Pourbakhsh et al. 1997a).

Previous observations on invasive capabilities of APEC have shown invasive capabilities of between 0.1 and 0.3% (La Ragione et al. 2000b; Ramirez et al. 2009), however these data were not obtained from DF-1 cells, rather they were obtained from primary tracheal culture, tracheal explant models and day-old-chick models. Further work is required to ascertain if 3770 can display an invasive phenotype in both cell culture and animal models.

3.3.4.3 – Survival inside phagocytes

As shown in Figure 9, persistence assays were performed to investigate the capability of strain 3770 to survive within avian macrophages. For all strains, survival decreased over time. 3770 decreased from 0.008% of the inoculum surviving at 1 hr to 0.004% at 2 hrs, 0.0008% at 4 hrs and 0% at 24 hrs. Strains UTI89, χ7122 and K12 were stable between 1 and 2 hrs, whilst APEC O1 exhibited a similar decrease to that seen in 3770 with a decrease from 0.005% of the inoculum surviving at 1 hr to 0.002% at 2 hrs and 0.0005% at 4 hrs. UTI89 had significantly higher levels of viable bacteria at 2 and 4 hrs ($P<0.05$)

Previous observations on the ability of ExPEC to survive within avian macrophage HD-11 cells revealed similar results, with a sharp drop in bacterial cell viability at 4 hrs and below detectable levels of bacteria at 12 and 24 hrs post infection (Gao et al. 2012).

Whilst APEC are known to have virulence factors that confer protection against phagocytosis, many of these gene products prevent phagocytosis from occurring in the first instance; cell surface factors such as *traT*, capsules, LPS and fimbriae prevent the initial recognition of the immune system or prevent opsonisation from occurring (Dziva and Stevens 2008).

It is possible that this prevention of phagocytosis is occurring in this infection model. We can observe the low levels of the inoculum taken up into the macrophages; less than 0.01% of the inoculum was phagocytised. 3770 possesses the K1 capsule (Timothy et al. 2008) which is strongly associated with prevention of phagocytosis, thus we could suggest that 3770 is able to evade aspects of the host immune system.

3.3.4.4 – Detection of inflammatory cytokines

RT-PCR was performed on DF-1 cells infected with an inoculum of 3770 to quantify the pro-inflammatory responses to infection with strain 3770. Three pro-inflammatory markers were investigated: CXCLi1, CXCLi2 and IL-1β.

The highest fold-change over all isolates was observed in the pro-inflammatory chemokine CXCLi2, with a fold change between 1.3-2.7 above the non-infected controls. The next highest fold-change was seen in IL-1β, with a fold change of between 0.6-1.66 above the non-infected controls. Finally CXCLi1 was observed with the lowest fold-change of between 0.18 and 1.8 fold-change above the non-infected controls. The strains that triggered the lowest inflammatory response overall were 3770 and χ7122. There was no evidence of an

inflammatory response specific to ExPEC. The only significant result was the production of CXCLi2 by K12 which was significant at ($P<0.01$).

An increase in CXCLi1, CXCLi2 and IL-1β has been observed following infections with *Campylobacter jejuni* and *S.* Gallinarum and Typhimurium (Kaspers and Kaiser 2014), and subsequent recruitment of polymorphonuclear cells would follow as the host attempts to phagocytise and clear the infection. As discussed earlier, bacteria such as ExPEC frequently possess virulence factors such as capsules that enable them to evade the host immune response (Reddick and Alto 2014). Two of the pathogenic isolates, 3770 and χ7122 provoked the lowest immune response of the 5 strains. As all 4 ExPEC isolates are encapsulated, this low immune response to 3770 and χ7122 is not likely to be caused by the molecular mimicry effects of a capsule alone. It is possible that 3770 and χ7122 possess a higher number of these immune-associated virulence factors than APEC O1 and UTI89, but further work would be required to support this theory.

3.3.5 – Chicken infection *via* inhalation route

The results in Figure 11 show the ability of 3770 to cause infection *via* the inhalation of aerosolised bacteria. χ7122 was used as an APEC comparison isolate, whilst K12 was used as a non-pathogenic control and fresh LB broth as a non-infected control. 3770 was able to infect the reproductive tract of 6/6 birds, with 100% positive for *E. coli* in the oviduct and 50% also positive in the ovules. No other systemic organs were infected by isolate 3770. In contrast, χ7122 was able to infect the heart, spleen and lung of one bird indicating the potential onset of colisepticaemia. χ7122 was also observed in the oviduct of 3/6 birds indicating that it is capable of causing reproductive tract infections. Both 3770 and χ7122 were significantly more capable of causing infection than the K12 and broth controls.

One bird in each of the K12 and the control groups were positive for *E. coli* in the oviducts and ovules. The detection of *E. coli* in the controls may reflect prior colonisation of the reproductive tract as these birds were fully mature and had been in production for around a year, or may simply be contamination during necropsy.

Previous infection models within the literature have shown successful infection in the reproductive tract *via* the intra-uterine inoculation route (Chaudhari and Kariyawasam 2014), *via* direct inoculation into the oviduct (Pors et al. 2014), and *via* the intravenous, intra-peritoneal, intra-tracheal, intra-vaginal and aerosol routes (Landman et al. 2013). In the aerosol infection model as described by Landman, the birds were exposed for 30 minutes to nebulized, aerosolised bacteria within an isolator. For the first time, this experiment showed that successful infections of the reproductive tract by SPS-associated bacteria such as strain 3770 could be induced *via* the use of a spray-based vaccine delivery system.

4 – Genome Sequencing of Strain 3770

The aim of this chapter was to undertake full genome sequencing on strain 3770 in order to provide detailed genomic information on an SPS-associated isolate of APEC. As of July 2016, there remain no published genome sequences for a reproductive-tract associated isolate of APEC, however an overview of the draft genomes of 2 reproductive-tract associated isolates has been published and these sequences are awaiting deposition into Genbank (Olsen et al. 2015).

4.1 – Introduction

The first *E. coli* genome was published in 1997 in *Science* (Blattner et al. 1997); a 4.6 megabase (Mb) sequence from a K-12 isolate. With a G+C content of 50.8% and a total of 4288 predicted genes, this K-12 genome sequence set the stage for further sequencing of *E. coli* genomes. As of July 2016 there are 97 annotated *E. coli* genome sequences deposited in Genbank, with a median total length of 5.16Mb and a G+C content of 50.6%. Modern NGS and bioinformatics techniques enable us to sequence an entire genome within a working week, however the *E. coli* K-12 sequence took a total of 6 years to complete using a combination of approaches as sequencing technology advanced rapidly during this time (Blattner et al. 1997).

The advances in NGS technology allow us to sequence multiple, entire genomes with high speed and accuracy, and low price. The advantages of which were clearly noticeable during the 2011 outbreak of O104:H4 in Europe, in which an unusual subtype of *E. coli* was responsible for a total of over 3000 cases of infection, of which there were over 800 cases of haemolytic-uremic syndrome and over 40 deaths. Within a week, a full genome sequenced was published and analysed using crowdsourcing techniques. The results indicated that the isolate in question was not a typical enterotoxigenic isolate; rather it was an enteroaggregative isolate which had acquired Shiga toxin. This case highlighted the use of rapid genome sequencing within a clinical setting, and also reminded

the scientific community how much more there is still to learn about the evolution of pathogenicity within this well-studied strain (Rohde et al. 2011; Mellmann et al. 2011; Gilmour et al. 2013).

4.1.2 – Next-Generation-Sequencing and *Escherichia coli*

The distributed genome hypothesis proposes that the genetic content of a species is larger than the genome of any one strain (Tettelin et al. 2005). This is particularly true of *Escherichia coli*. Genome dynamics within *E. coli* are highly variable and flexible, with a range of diverse adaptations to different hosts within the species. Estimates of the percentage of core genome common to all strains differ slightly between studies however there is agreement that the core genome is overall a low percentage (6-10%) of the total genome within each *E. coli* strain (Rasko et al. 2008; Touchon et al. 2009; Lukjancenko et al. 2010) and recombination events are high (Touchon et al. 2009). The widespread horizontal transfer events result in distinct islands within the genome, often called genomic islands or pathogenicity islands. The pan-genome of *E. coli* has been called 'open' due to on-going gene acquisition (Rasko et al. 2008).

Since the early days of genome sequencing, there has been a strong focus on what we now call 'pathogenomics' or the specific genomic elements associated with pathogenicity with the aim of using our increased knowledge against the bacteria with targeted pan-species vaccines or drugs. There is still a large volume of unknown knowledge on a genetic level relating to the more mundane cellular processes and as such, many sequenced genomes contain predicted genes based on orthology to other genes, homology to a functional family, or even unknown open reading frames (ORFs) with unknown function which unfortunately still limits our overall knowledge and ability to use rational drug design techniques (Dobrindt 2005).

The genome sequence of the APEC O1:K1:H7 isolate (referred to as APEC O1) was published in 2007; the first APEC isolate to undergo full genome sequencing

and part of the ST95 lineage (Johnson et al. 2007). Since then, more APEC genome sequences have been published, which will be discussed below. The study by Johnson et al. tested the hypothesis that certain APEC strains have potential to cause urinary tract infections in humans. By comparing the complete genome sequence of APEC O1 to all available human ExPEC genome sequences they discovered that the genome of APEC O1 was 'remarkably similar' to three sequenced UPEC isolates, by including MLST in the analysis it was also revealed that some sequenced human ExPEC isolates were more similar to APEC O1 than they were to other human ExPEC strains. The size of the APEC O1 genome was 5.6Mb including 4 large plasmids, which have separately been characterised and will be discussed shortly in Section 5.1.2.1. Overall, only 4.5% of the APEC O1 genome was not found elsewhere within the collective ExPEC genome. The G+C content of APEC O1 was observed to be similar to that seen in other sequenced *E. coli* at 50.5% however regional differences were observed in the genomic islands. Notable virulence factors were observed, such as the pap operon, numerous iron transport systems, *iss,* vacuolating auto transporter, temperature sensitive haemagglutinin and invasins such as *ibeA* and *tia* (Johnson et al. 2007).

Two APEC O78 type isolates were sequenced by Dviza et al. in 2013, in order to study an ST lineage of APEC; ST23 (Dziva et al. 2013). The two isolates within this study were χ7122 and IMT2125, for the purpose of this thesis; only χ7122 will be discussed here. This study noted that the ST23 isolates were more closely related to human enterotoxigenic isolates than they were to APEC O1. χ7122 was revealed to have a chromosomal genome 4.7Mb in size with 4 large plasmids adding an additional 2.50kb, encoding a predicted 4627 genes in total. There was a low degree of similarity between χ7122 and APEC O1 in terms of the genomic islands and pathogenicity islands. APEC O1 possessed large regions containing multiple virulence genes and loci that were not observed in χ7122. Further experimentation with signature-tagged-mutagenesis revealed key roles in virulence for genes present in χ7122 but absent in APEC O1, such as the group 4 capsule synthesis operon, flagella and Yad fimbriae, and

the alternative iron uptake genes *entF* and *yddB.* The study concludes that APEC are not uniformly related to ExPEC, since the two APEC strains at the focus of this study were clustered with human enterotoxigenic *E. coli* strains. They did not suggest that these APEC strains would be capable of causing diarrheal disease in humans, as they lack any of the key enterotoxins, however that mutations in the core genome of ST23 can give rise to mutants able to cause disease in avian species (Dziva et al. 2013).

A paper by Olsen et al. describes draft genome sequences of three strains of avian *E. coli*; one of which is a faecal commensal isolate, and two were isolated from reproductive tract infections. Of the two strains originating from reproductive tract infections, one was isolated from a monoclonal outbreak in the field, whilst the other was isolated from a clinical case of salpingitis. The three sequenced genomes were revealed to have genomes of 5Mb in size, or 5.5Mb in the case of the faecal commensal isolate. All three isolates were annotated and predicted to have in the region of 5000 putative genes, of which both of the strains associated with reproductive tract infections were highly similar in terms of their virulence content; each positive for 32 and 37 out of 58 virulence genes analysed. The isolate associated with the monoclonal outbreak possessed the highest number of virulence genes, differing from the isolate originating from the clinical case of salpingitis by possession of *iutA, papA, tia, iucB* and *iucC*. Use of the bioinformatics tool PathogenFinder was used to estimate the number of pathogenic families associated with each genome. For all three isolates, this analysis predicted pathogenicity in humans. Additionally, for the isolate associated with the monoclonal outbreak, 959 pathogenic families were observed within the genome revealing high levels of predicted virulence. The genome sequences of these isolates still remain to be deposited in Genbank (Olsen et al. 2015).

4.1.2.1 – Plasmids

Plasmids have long been associated with virulence in APEC (Smith and Huggins 1976), and as our knowledge increases so does the strength of this association with virulence.

A study in 1996 was the first to examine and compare a large number of plasmids from APEC. In the study, 30 isolates obtained from a mixture of healthy and sick birds underwent plasmid extraction, and it was observed that 17 of these isolates had plasmids greater than 50kb in size and of these 17 isolates, 11 had plasmids greater than 100kb in size. Some of the larger plasmids contained a region with homology to the colicin V gene; *cvaC,* and these isolates were more frequently derived from sick birds than healthy chickens, however the production of colicin was not a factor involved in virulence; merely a marker (Doetkott et al. 1996).

10 years later, a ColV plasmid was fully sequenced for the first time. It was revealed to be a 180kb plasmid with a large 93kb region containing many APEC virulence factors such as *iss*, *tsh*, and 4 iron-uptake systems. To further study the prevalence of this plasmid, a population of 595 APEC and 199 faecal commensal bacteria were studied for both the prevalence of this plasmid and the degree of conservation of this plasmid within the population. The 93kb cluster of virulence genes was shown to be well conserved within the population, and as such it was suggested that this region of DNA be a distinguishing trait of APEC subtypes (Johnson et al. 2006c).

Shortly after, the Johnson lab published a second paper detailing the DNA sequence of a ColBM plasmid, also obtained from an APEC isolate. In this study they describe a second, related plasmid 174kb in size with a similar virulence cluster described in the previous ColV paper. They suggest that ColBM plasmids themselves derive from ColV plasmids, describing remarkable similarity and remnants of the ColV operon within the ColBM plasmid which itself encodes for

colicins B and M. This study provides further support to the suggestion that the conserved region of virulence genes is strongly linked to virulence in APEC (Johnson et al. 2006b).

Further evidence that the ColV/BM plasmids are strongly associated with APEC virulence was provided in a study in which a commensal *E. coli* isolate underwent modification to acquire a ColV plasmid. Within a chick lethality assay, the modified strain and a positive control were significantly more lethal than the original plasmid-negative strain. It was also more virulent in a mouse model of ascending urinary tract infection, suggesting that these plasmids could also play a role in human infections (Skyberg et al. 2006).

More recently, a study investigating the contribution to virulence of 3 large plasmids found in the O78 χ7122 isolate showed the individual contribution to virulence of each plasmid. Unsurprisingly, the loss of all 3 plasmids severely attenuated the virulence of strain χ7122, whilst the presence of different plasmids and combinations of different plasmids generated a range of mutants with varying pathotypes and levels of virulence (Mellata et al. 2010).

4.1.2 – Virulence Genes

A variety of virulence genes have been identified and characterised within ExPEC populations and APEC subpopulations. These have been described extensively in section 1.3.

To provide a brief overview, virulence genes within ExPEC populations are associated with the following functions:

a) Iron acquisition systems
b) Factors to enable evasion or modulation of the host immune response
c) Toxins
d) Cell surface structures
e) Factors enabling attachment to host cells, and or invasion of host cells

Research into virulence factors within APEC populations has long focused on the search for a concrete APEC pathotype. However, study after study has revealed high variation within this population (Ewers et al. 2004; Rodriguez-Siek et al. 2005a; Johnson et al. 2008a; Maturana et al. 2011; Kemmett et al. 2013; Collingwood et al. 2014), with no single gene responsible for pathogenicity. It tempting to speculate that virulence is more likely a complex relationship between the virulence profile of the bacterium and the health status of the host rather than a specific APEC pathotype (Collingwood et al. 2014).

4.1.3 – Phylogenetics of ExPEC

Typing of *E. coli* has been largely based on serotyping based on the O (LPS or somatic antigen), H (flagella) and K (capsule) antigens. As with other species, most notably *Salmonella*, there is an increasing use of molecular-based typing which is considerably less subjective and frequently more informative of the relationship between isolates (Achtman et al. 2012).

Multi-locus sequence typing uses the genetic sequences of between 6-8 housekeeping genes in order to observe small, natural variations within populations and to obtain a 'sequence type' (ST). Common STs in APEC populations are ST131, ST393, ST69, ST95 and ST73 (Riley 2014).

Phylogenetic typing in ExPEC populations has shown conflicting data within the overall phylogenies. Depending on the methods used, there can be strong links between phylogeny and virulence, (Picard et al. 1999; Johnson et al. 2006b; Moulin-Schouleur et al. 2007). Other phylogenetic studies however show that there can be strong variances between trees created using different methodology indicating that recombination events may be obscuring the true phylogenetic relationships between strains (Johnson et al. 2006a).

Construction of whole-genome phylogenetic trees has been shown to reduce biases and differences due to choices in housekeeping genes or quality of data.

It also suffers from little impact caused by the high degree of recombination present in *E. coli* populations (Touchon et al. 2009). This method generates consistent trees, however the methodology is more complex than creation of phylogenetic trees based on one or two genes, as is the more usual method (Chaudhuri and Henderson 2012).

4.1.4 – Metagenomics and ExPEC

Advances in NGS have created possibilities in metagenomics that could have only been dreamt about 10 years ago. Comparative sequencing of more than 100 isolates in one study is now by no means unusual.

A recent study by Salipante et al. performed whole-genome sequencing on 312 human ExPEC isolates derived from sepsis or UTI at a single healthcare institution. Of the isolates that underwent sequencing, 283 were deemed of high enough quality to be used in the analysis. The pan-genome for the isolates within this study was an estimated 14,877 genes once insertion sequences and prophage genes had been removed. Of these 14,877 genes, 3039 genes were classed as the core genome, which was somewhat higher than the expected 10% observed in other studies as mentioned previously. As expected the core genome contained many genes related to basic cellular functions whilst the pan-genome contained many genes classed as virulence factors with functions such as capsule synthesis, iron acquisition, secretion systems and flagella. Interestingly there was no specific gene present in the pathogenic population that was not also present in a high frequency in commensal populations indicating that, at least in this particular population there is no specific gene required for virulence. This population was highly clonal, with 71 different MLST types observed within the population, split evenly between established STs and novel STs. Isolates were also split amongst the different ExPEC phylogroups, with the largest group, B2, comprising 65.7% of the population. These phylotypes also unsurprisingly possessed the largest number of virulence genes (Salipante et al. 2015). The disadvantages of this study in terms of its relevance

for clinical sequencing of bacterial isolates is that no novel antibiotic resistance factors were observed within any of the 283 isolates, nor any evidence of patient-to-patient transmission. For the purposes of examining the original source of an infection or identifying new antibiotic resistance markers, this study does not appear to support the routine usage of genome sequencing in a clinical setting.

Whilst the advantages to NGS are numerous, and the improvements in technology exciting – there still exists a large problem preventing us from truly benefitting from the potential inherent in NGS. We are limited in several factors, and until these are overcome there will be large problems within the field of metagenomics and also within the clinical usage of genomics and bioinformatics. Our lack of knowledge on the function of many genes means that we cannot fully characterise large regions of genomes. Difficulties in extracting DNA can mean that metagenomics studies don't truly reflect the target population but rather more those microorganisms with more easily obtained DNA. Assembly and analysis methods are still in need of improvement too, in particular, the computing power needed for large metagenomics studies ensures that it is out of reach for smaller research institutions (Scholz et al. 2012).

4.1.5 – Illumina Sequencing Technology

The Illumina NGS platform performs massively parallel sequencing which allows us to sequence an entire genome within a day at a low cost enabling many labs to own a MiSeq system.

Similar to many other methods of genome sequencing, initially a library is created and amplified. The MiSeq platform uses glass flow cells, and amplification is achieved using a bridging method. The library DNA acts as templates for the longer DNA fragments to be synthesised. Buffers containing one type of fluorescently labelled reversible-terminator nucleotides are

sequentially washed over the flow well, and detection of the fluorescence patterns is digitally captured and converted into a raw DNA sequence (Quail et al. 2012).

The raw DNA sequence must undergo initial quality control and analysis including removal of low-quality reads and adaptor sequences. Once this has been completed, the sequence can be 'scaffolded' onto an existing reference genome (in which the reference genome is used as a template for assembly) or *de novo* alignment can be performed if required (Grada and Weinbrecht 2013).

4.2 – Results

4.2.1 – The 3770 genome is 5.02Mb in size

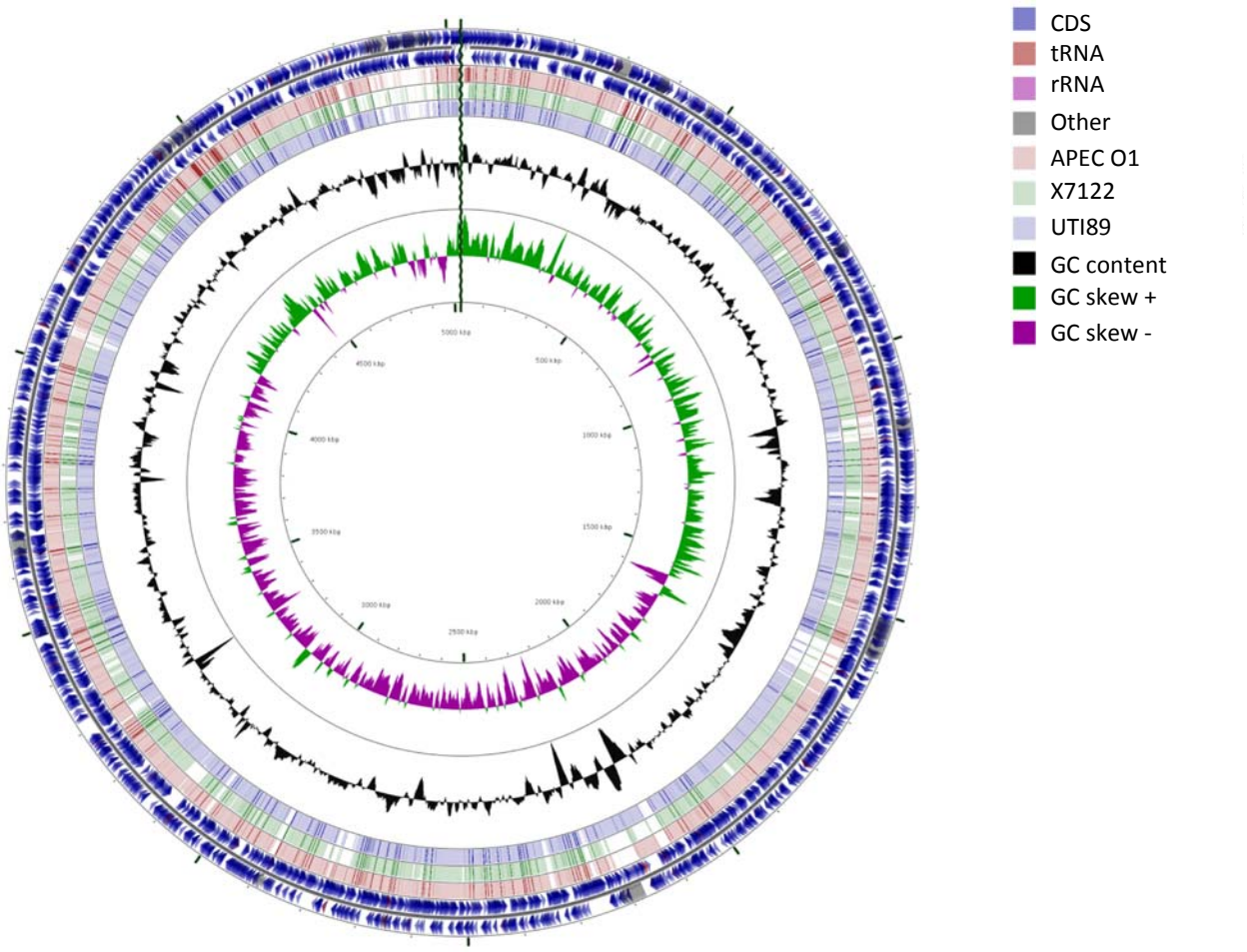

Figure 12: Map of the 3770 chromosome and comparison of 3700's chromosome to those of other ExPEC strains.

In this CGView image the 3770 genome is aligned and compared to the genomes of APEC O1, UTI89 and χ7122. The outer two rings are coding regions in forward and reverse orientation, including tRNA, rRNA and other regions. The 3rd ring shows the genome alignment of 3770 with APEC O1 (light red), the 4th ring shows the genome alignment of 3770 with X7122 (light green), and the 5th ring shows the genome alignment of 3770 with UTI89 (light purple). The 6th ring shows GC content compared to the overall average, with the 7th ring showing positive skew and the 8th ring showing negative skew.

4.2.2 – The genome of 3770 shows similarity to other ExPEC genomes

The 3770 genome was assembled at the Centre of Genomic Research, University of Liverpool, from 74 contigs using a comparison with χ7122 to order the contigs and prepare a scaffold, which then underwent annotation using Prokka with a custom database of all available annotated *E. coli* and *Shigella* genomes.

The 3770 genome is 5.02Mb in size, which is slightly smaller than the average *E. coli* genome, 5.16Mb, as stated by Genbank. There were no plasmids detected. There are 4671 predicted genes within the 3770 genome and there are 78 tRNA coding sequences. The percentage content of each nucleotide within the genome is as follows: A: 24.25%, C: 24.38%, G: 27.23%, T: 24.11%. Overall the G+C content of 3770 is 50.52%, which is consistent with the average G+C content of *E. coli* of 50.6%, as calculated by Genbank.

CGView (http://wishart.biology.ualberta.ca/cgview/) was used to generate a circular genome alignment with APEC O1, χ7122 and UTI, as shown in Figure 12. There are overall very strong similarities between the ExPEC genomes, as to be expected when comparing closely related ExPEC isolates. However, there are notable areas within the 3770 genome that are not contained in the comparison genomes. These are visible in the gaps in the light red, light green and light purple rings (rings 3, 4 and 5), in which the sequence of 3770 contains nucleotides the comparison genomes do not.

The G+C content is consistent with the average *E. coli* G+C content as noted on Genbank, which is 50.6%. Interestingly the skew of the G+C content within the 3770 genome as compared to the genomes of APEC O1, X7122 and UTI89 is split, with a strong positive skew for approximately the first 1500kbp and the last 900kbp, and a strong negative skew between 1500kpb and 4100kbp.

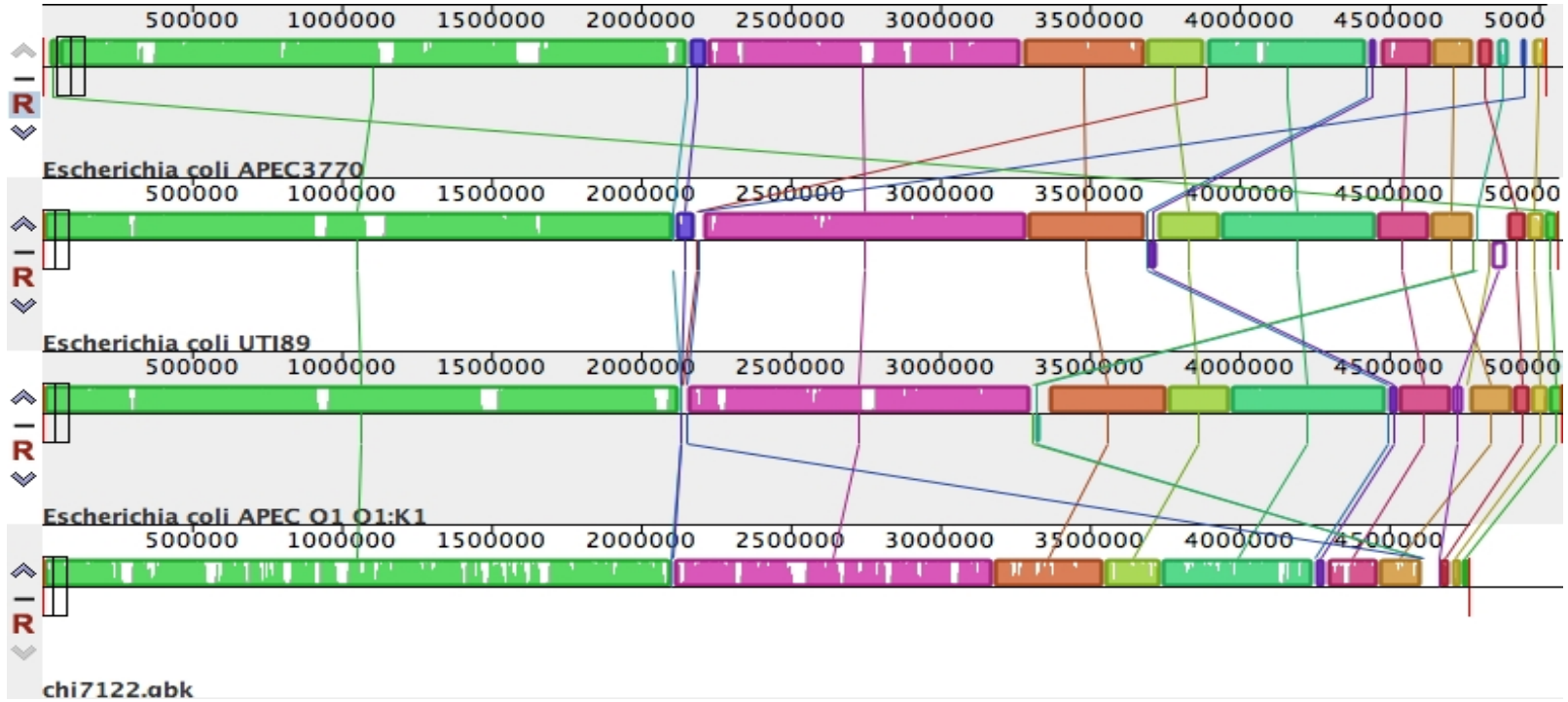

Figure 13: ProgressiveMauve alignment of 3770 with UTI89, APEC O1 and χ7122.

This alignment shows large-scale changes between the genomes such as gene loss, duplication, rearrangement and horizontal transfer. Each coloured segment represents a region of DNA that is conserved across the 4 genomes. White sections within the segment represent insertions and deletions. Lines between the genomes represent sections of DNA, which appear in different regions within the chromosome in different strains. Segments below the lines indicated inverted sequences

4.2.3 - ProgressiveMauve analysis revealed similarities between 3770, UTI89 and APEC O1

The genome of strain 3770 was aligned against UTI89, APEC O1 and χ7122 using ProgressiveMauve as shown in Figure 13.

Between bases 0 and 2Mb there is a very high degree of similarity between all 4 isolates, as shown by the first green segment. White sections within the segment shown regions in which the genome content differs due to insertions and deletions.

After 2.25Mb the degree of similarity between 3770 and the comparison strains decreases. UTI89 remains the most similar, with concordant regions throughout the genome.

APEC O1 has largely similar conserved regions, however as displayed by gaps and sections beneath the line there are regions in 3770 that are not in APEC O1 and there are also regions that appear in different sections within the chromosome.

χ7122 has the least degree of similarity. On a superficial level, there are similarities within the overall regions however these are marked with insertions and deletions and a markedly shorter genome size.

4.2.4 – Comparison between 3770 and χ7122 genomes

The Artemis Comparison Tool (ACT) (Carver et al. 2005) was used to compare the genomes of 3770 and χ7122. According to this alignment the largest regions of variation between the genomes include a 1.5Mb region between bases 1,586,000 and 1,657,500. This region is not observed in χ7122 however in 3770 this region contains a number of coding sequences identified as originating from

P1 phage such as the *pacAB* phage packaging proteins. Further comparison of this region using BLAST revealed a 72.5kB prophage located near tRNA-Met, tRNA-Thr and tRNA-Asn. This region was not observed in other sequenced *E. coli* isolates however there was similarity to two *E. coli* plasmids and P3 and P7 bacteriophages.

In addition, a region between 294,800 and 325,600 was observed in the 3770 genome that was not observed in the χ7122 genome. In the 3770 genome this region is located near tRNA-Asp and contains a cluster of genes associated with a type VI secretion system. BLAST searches revealed that this was the Type 6 secretion system 2 loci (T6SS2) which is found in numerous other *E. coli* sequences including APEC O1 and UTI89 but not χ7122 and is associated with B2 phylogenetic group (Ma et al. 2013).

4.2.5 – Whole-genome phylogenetic typing showed dissimilarities between 3770 and other ExPEC such as APEC O1 and UTI89.

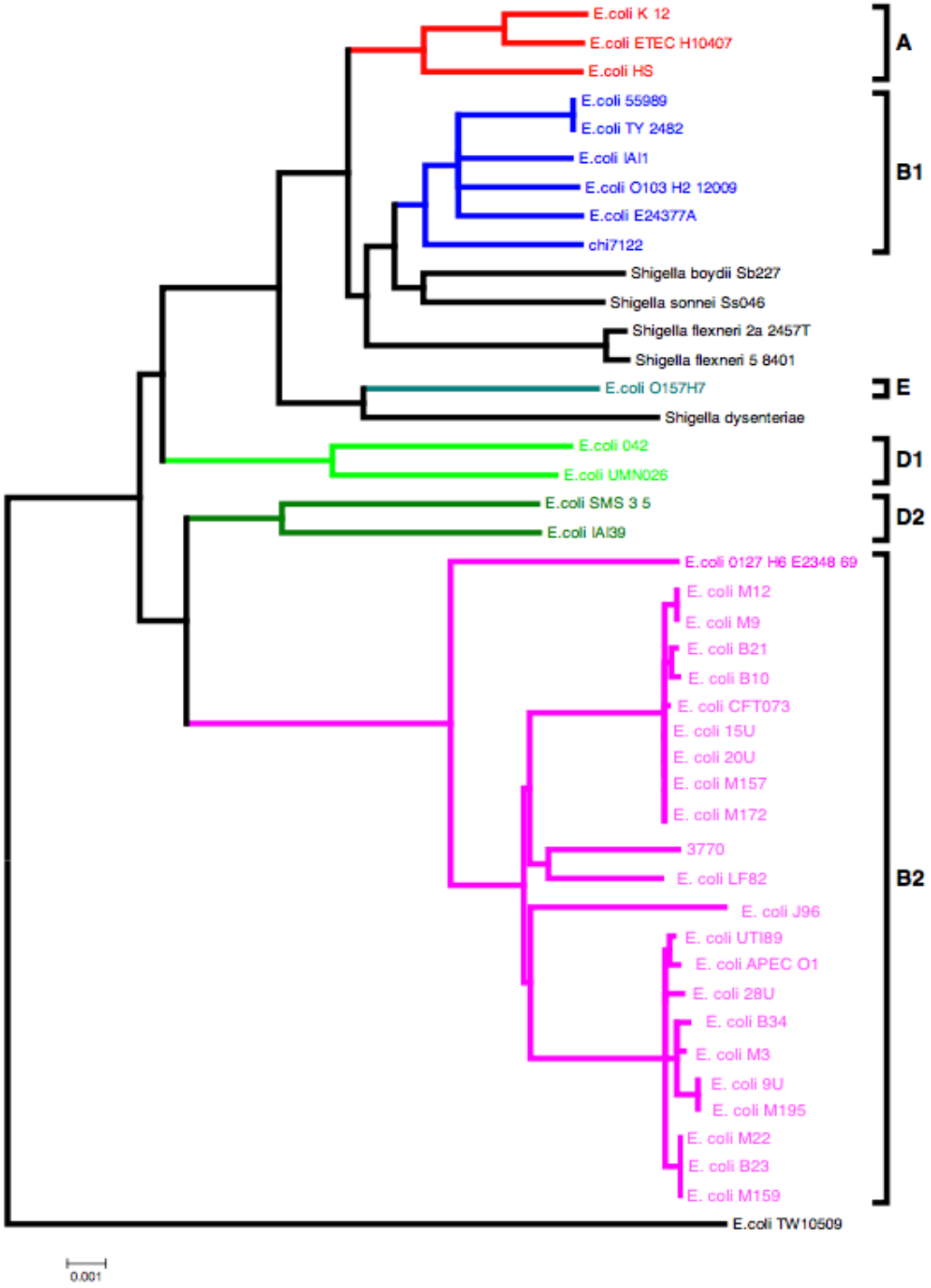

Figure 14: A whole-genome phylogenetic tree of *E. coli* database

A whole-genome alignment was created in Mugsy and a phylogenetic tree created using the neighbour-joining algorithm in ClustalW. Isolates are shown clustered into the characteristic *E. coli* phylogenetic groups.

In order to examine the phylogenetic grouping of 3770 within other *E. coli* a whole-genome phylogenetic tree was constructed using a custom *E. coli* database containing 43 fully sequenced *E. coli* and *Shigella* genomes. This alignment was created in Mugsy, then the neighbour-joining algorithm was used in ClustalW to create a whole-genome phylogenetic tree.

Isolate 3770 was found to be in the B2 phylogenetic group, alongside APEC O1 and UTI89. χ7122 is found in the B1 phylogenetic group.

In this analysis of whole genomes, isolate 3770 is most closely related to *E. coli* isolate LF82, which is an adherent-invasive subtype of *E. coli* associated with Crohn's disease (Miquel et al. 2010). This analysis showed that 3770 and LF82 are clustered together within the B2 phylogenetic group having diverged earlier in their evolution than UTI89 or APEC O1.

ST	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	ST Complex
ST141	13	52	10	14	17	25	17	None
ST141	**13**	**52**	**10**	**14**	**17**	**25**	**17**	**None**

Figure 15: MLST sequence type.

MLST was performed using the sequences of *adk*, *fumC*, *gyrB*, *icd*, *mdh, purA* and *recA* as obtained from the nucleotide sequences from the 3770 genome sequence. 3770 was shown to belong to ST141.

4.2.6 – 3770 has a large variety of virulence genes

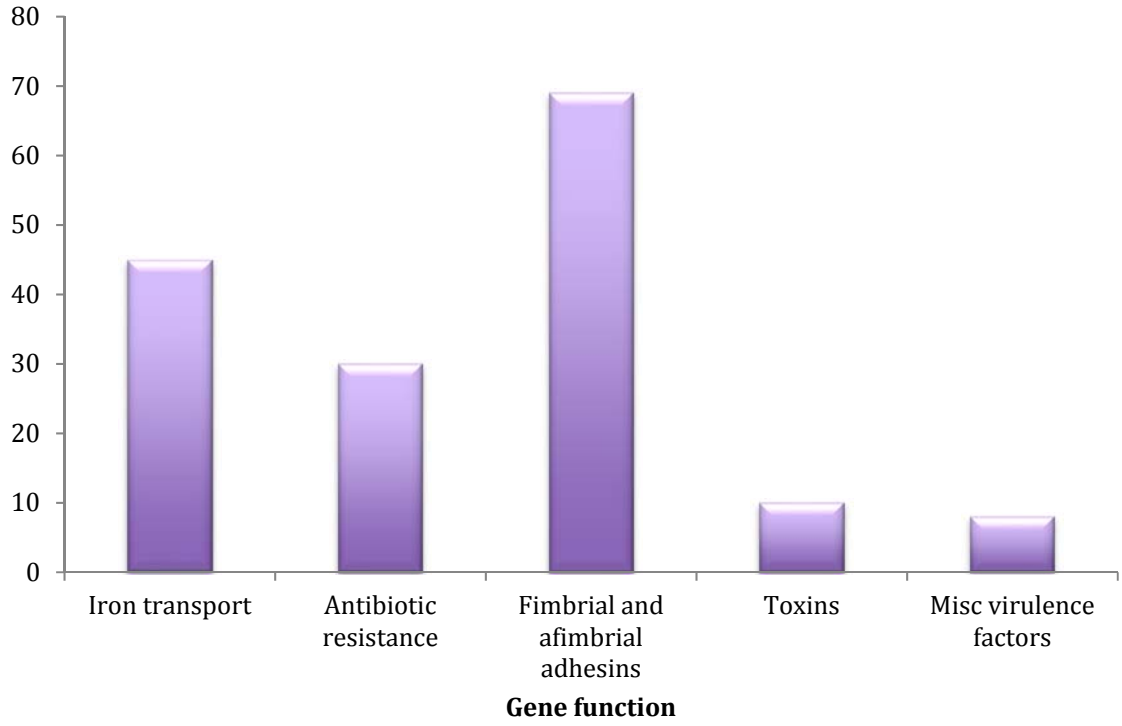

Figure 16: 3770 virulence genes by function

The number of genes associated with virulence was calculated and displayed as a graph. Virulence genes are broken into broad functional classes; iron transport, antibiotic resistance, fimbrial and afimbrial adhesins, toxins, and miscellaneous factors such as *iss* and invasins.

The genome of strain 3770 was investigated for the prevalence of various APEC virulence factors, which are shown as functional categories in Figure 16.

Within the 3770 genome, there are 162 genes associated with virulence functions such as iron transport systems, antibiotic resistance, fimbrial and afimbrial adhesins, toxins and miscellaneous factors such as invasins.

Within the category of iron transport, there are 45 genes associated with this function including well characterised genes and operons such as the *sitABCD* locus, which appears twice within the genome, the yersiniabactin operon, *iucABCD*, *iroN* and *iutA*.

Within the category of fimbrial and afimbrial adhesins 69 genes were observed associated with these functions, including operons encoding flagella, lateral flagella, fimbriae, S fimbriae, and the putative *auf* fimbrial operon.

Within the category of toxins, 10 genes were observed. These included *hlyF*, *tsh*, *vat* and putative toxin-antitoxin genes.

Within the category of miscellaneous virulence factors, a total 8 genes, was observed including *iss,* the *ibeABCT* operon, *ibrB* and K1 capsule genes.

Genes	Gene product	Function
sitABCD	sit proteins	Fe/Mn uptake
chuAUV	haem-binding protein	Iron uptake
iucABCD, iutA	aerobactin	Iron uptake
iroBCDEN	salmochelin	Iron uptake
entABCDEF, fepABCDE	enterobactin	Iron uptake
ybtSXQEAUTE	yersiniabactin	Iron uptake
feoABC	ferrous iron uptake	Iron uptake
hlyF	haemolysin F	Toxin
tsh	temperature sensitive haemagglutinin	Toxin
vat	vacuolating autotransporter toxin	Toxin
iha	Adhesin	Adhesin
sfaAGS	S fimbriae	Adhesin
ibeABCT	Invasin	Invasin
kpsMTSCUDEF, neuDBACES	K1 capsule	Cell surface
iss	Increased serum survival	Cell surface

Table 3: APEC virulence factors are prevalent within the 3770 genome

3770 was shown to be positive for many virulence genes associated with APEC pathotypes as shown in Table 3, above.

The 3770 genome contains 4 siderophore operons; aerobactin, salmochelin, yersiniabactin and enterobactin. Additionally, 3770 was positive for the *feo* ferrous iron uptake system, the *chu* haem binding protein and the *sitABCD* locus, which appeared twice in the genome.

3770 was positive for the toxin-encoding genes *hlyF*, *tsh*, and *vat.*

3770 was positive for many adhesins, as seen on Figure 19. In particular, *iha* and *sfaAGS* were positive. Invasin was also observed, with the full *ibeABCT* locus present.

3770 was positive for cell surface factors including the entire K1 operon and *iss*.

4.2.7 – PathogenFinder analysis

PathogenFinder was used to analyse the entire genome of 3770 to assess predicted pathogenicity according to a protein family database composed of groups of proteins from both pathogenic and non-pathogenic bacteria (Cosentino et al. 2013).

Strain 3770 was predicted to be a human pathogen with a probability of 0.932 of a maximum probability of 1.0. A total of 701 pathogenic protein families from the database were matched, compared to a total of 5 non-pathogenic families matched. This indicates that pathogenicity in humans would be possible for strain 3770. As this strain is known to be pathogenic in avian species, and APEC are known to be highly similar to isolates pathogenic in humans (Ewers et al. 2007) this result is not surprising.

Matches were reported for 198 *E. coli* UTI89 gene sequences, 166 *E. coli* 536 gene sequences, 62 *E. coli* CFT073 gene sequences, 31 *E. coli* APEC O1 gene sequences, 28 *Escherichia coli* ED1a gene sequences, 27 *E. coli* E24377A gene sequences and other *E. coli* strains were also observed with less frequency. Interestingly, 111 gene sequences were closely matched to *Shigella* species.

4.2.8 – CARD analysis

The Comprehensive Antibiotic Resistance Database (CARD) bioinformatics tool was used to analyse the genome of strain 3770. The CARD contains information on antibiotic resistance genes, gene products, and associated phenotypes (Jia et al. 2017).

The CARD analysis of the genome of 3770 revealed matches to 238 antibiotic resistance genes and mutations within the database of which 120 were matches to regions found in *Escherichia coli* species. Resistance phenotypes were noted for a large variety of antibiotic types including beta-lactams, aminoglycosides, tetracyclines, rifamycins, fluoroquinolones, and sulphonamides.

4.2.9 – Presence of ETT2

The 3770 genome was analysed for the presence of the ETT2 gene cluster by manual analysis of the genome searching for genes and primers sequences as described previously (Ren et al. 2004). The upstream region containing genes *araE, kduDI* and *yqeF* was observed, however no further genes or matching primer sequences were found within the genome.

4.3 – Discussion

The sequencing of the 3770 genome provides insight into the SPS-subtype of APEC infections, of which there is no published annotated genome sequence. *Via* comparisons to published APEC and UPEC isolates, we have been able to ascertain that 3770 has a genome 5.02Mb in size, with a G+C content of 50.52%. These values are similar to the data seen elsewhere in the literature (Johnson et al. 2007; Touchon et al. 2009; Lukjancenko et al. 2010; Dziva et al. 2013; Olsen et al. 2015) thus showing that on a genomic level, 3770 is characteristic of the *E. coli* group.

4.3.1 – 3770 is similar to APEC O1 and UTI89 on a whole-genome scale

3770 shows high concordance with APEC O1 and UTI89 when examined on a whole-genome scale. Figure 12 allows us to consider the entire circular genome alignments of 3770, UTI89, APEC O1 and χ7122. In this figure we can clearly see both large regions of similarity interspersed with smaller areas of sequence divergence in which features have been gained or lost. Similarity to UTI89 was the highest, with conserved regions of similarity visible in the Mauve alignment in Figure 13. Concordance with χ7122 is lower with many variations visible in the conserved regions, less homology with the conserved regions overall, and a much shorter genome as shown in the Mauve alignment in Figure 13.

The APEC O1 genome is 5.6Mb in size including 4 large plasmids (Johnson et al. 2007), however no plasmids were observed in the sequence of 3770. The ColV/BM plasmids have been suggested to be distinguishing of APEC virulence (Johnson et al. 2006c, b), however the lack of plasmids in this virulent isolate suggests that plasmids are not a defining characteristic. The virulent 93kb region within the ColV/BM plasmid contains virulence factors such as *iss, tsh* and 4 iron-uptake systems including salmochelin, aerobactin and the *sit* locus,

all of which are found, separately, within the 3770 genome indicating that these genes may also be found within the chromosome and that these genes themselves might be more indicative of virulence than the presence of plasmids.

4.3.1.1 – Impact of scaffolding genome onto χ7122

It has previously been observed that the differences between APEC O1 and χ7122 are high, with limited similarities between genomic islands, virulence associated loci, and even different lineages (Dziva et al. 2013). The use of this reference genome as a scaffold for the assembly of strain 3770 could cause limitations in the accuracy of the assembled 3770 genome due to the potential unsuitability of χ7122 as a reference genome for APEC isolates. The assembly was created prior to the publication of the χ7122 genome in 2013, and as such the large variation between the two APEC genomes was not known. It would be beneficial to reassemble the 3770 genome using APEC O1 as a reference genome, and to compare the two resulting genomes.

4.3.1.2 – Comparison and variation to χ7122

Two notable and interesting regions of difference were observed during analysis with ACT (Carver et al. 2005) which then underwent further BLAST analysis. Investigation revealed a region near tRNA-Met, tRNA-Thr and tRNA-Asn which contained a prophage which had some similarity to plasmids found in *E. coli* O111:H- and O55:H7, and also similarity to P3 and P7 phages. Further investigation using a plaque assay could show if these phages are able to replicate.

An additional region near tRNA-Asp contained the Type 6 secretion system 2 loci (T6SS2) which is found in numerous other *E. coli* sequences including APEC O1 and UTI89 but not χ7122 and is associated with B2 phylogenetic group (Ma et al. 2013). This secretion system has previously been shown to be linked with

virulence, potentially with BMECs (Ma et al. 2014), indicating a potential for cerebral infection by strain 3770. This was not investigated during the infection experiment, however it could be an interesting avenue for future work.

4.3.2 – 3770 is not a common sequence type, and diverges from other ExPEC on a whole-genome phylogenetic tree.

MLST was performed on strain 3770 as shown in Figure 15. The sequence type was shown to be ST141. This ST has been previously observed in APEC populations (Olsen et al. 2011; Pires-dos-Santos et al. 2013), however it is not a commonly-seen ST nor is it strongly associated with virulence. Whilst clearly 3770 is a pathogenic isolate, we are not able to infer any further information from MLST data. There have been criticisms of MLST in the past, in particular that the arbitrary choice of housekeeping genes could bias the results. It has been noted that mutations in one of the MLST typing genes, *gyrB*, are associated with quinolone resistance so it could in fact be suggested that this gene is under selection pressure to mutate and therefore would not be a strong candidate for a conserved gene (Chaudhuri and Henderson 2012).

A whole-genome tree was created using Mugsy and the neighbour-joining algorithm in ClustalW, as shown in Figure 14. In this phylogenetic tree, we can see that strain 3770 is most similar to LF82 which is an adherent-invasive (AIEC) strain of *E. coli* associated with Crohn's' Disease. This was an unexpected result, given that LF82 is not a member of the ExPEC family. AIEC are known to colonise ileal lesions in Crohn's' disease patients, in which they are able to replicate within intestinal epithelial and macrophage cells. The genome sequence of LF82 was published in 2010, and it was noted that this strain was unexpectedly similar to APEC O1 and UTI89 despite not possessing most of the more common ExPEC virulence factors. Additionally, it was shown that UTI89 and LF82 were similarly able to adhere to intestinal cells *in vitro.* The authors of this paper suggest that that LF82 evolved *via* acquisition of *Salmonella* and

Yersinia gene clusters (Miquel et al. 2010). In the whole-genome phylogenetic tree constructed in Figure 14, 3770 and LF82 are clustered together and diverged separately from other group B2 isolates such as UTI89 and APEC O1. It is therefore also possible that 3770 evolved in a similar manner, by gaining gene clusters from related species such as *Shigella* and *Salmonella.* There is a precedent within the literature for a similar evolution towards an APEC pathotype from an enteropathogenic isolate; χ7122 was shown to be more closely related to a human enterotoxigenic *E. coli* than to other ExPEC and it was suggested that the core genome allowed for adaptation towards different pathotypes *via* the acquisition of distinct virulence genes (Dziva et al. 2013).

4.3.3 – Virulence gene content of 3770 was high

Overall virulence content within 3770 was high, with a total of 162 genes associated with virulence factors such as adhesins, antimicrobial resistance factors, toxins and iron uptake systems.

Iron uptake systems were particularly high in 3770, with possession of a total of 45 genes associated with iron uptake, as shown in Table 3, including the full operons for all 4 siderophore systems found in ExPEC; enterobactin, aerobactin, yersiniabactin and salmochelin, of which the gene loci are shown below in Figure 17. In addition, the *sitABCD* locus was found twice within the genome.

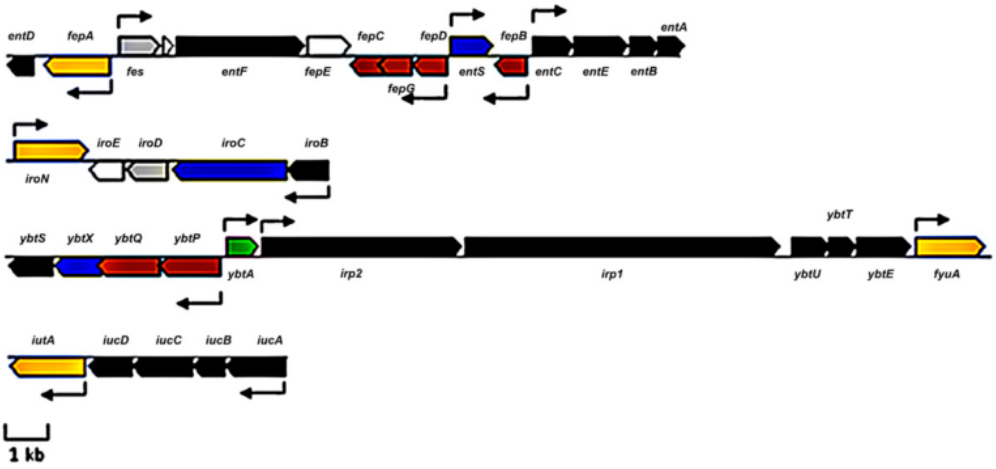

Figure 17: The 4 genetic loci of the siderophore systems found in ExPEC

(Garénaux et al. 2011)

Possession of iron uptake systems has been shown to be important for ExPEC isolates. Studies using deletion mutants have clearly shown decreased virulence in a variety of models when iron uptake systems are attenuated (Garcia et al. 2011; Gao et al. 2012), and that the iroN receptor contributes to invasion of urothelial cells *in vitro* (Feldmann et al. 2007), and additionally that the *sitABCD* locus contributes towards hydrogen peroxide resistance (Sabri et al. 2006) which could confer protection against phagocytosis.

The K1 capsule operon was fully present in 3770, as shown in Table 3, and the full operon is as illustrated below in Figure 18.

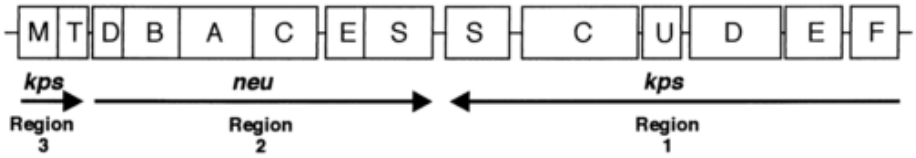

Figure 18: the genetic locus of the K1 capsule consists of 14 genes

(Bliss and Silver 1996)

The study on 3770, observed the presence of *kpsMT* (Timothy et al. 2008), and simple testing showed that the K1 capsule was present in strain 3770. The K1 capsule comprises sialic acid residues, which allow the bacterium to evade the host immune system by mimicking host structures. It has previously been implicated as important for both human and avian diseases (Brée et al. 1989; Dho-Moulin and Fairbrother 1999; Kim et al. 2003; Ewers et al. 2007) and as such it is likely that possession of the K1 capsule would provide an advantage within the host environment and allow strain 3770 to evade the host immune response.

Possession of fimbrial and afimbrial adhesins was high, with a total of 69 genes associated with this function. There is a consensus within the literature that the ability to adhere to host cell surfaces is the most important step in colonisation of a host; without the ability to adhere, a bacterium is unable to secure a niche

for itself within the host environment. Many fimbrial and afimbrial adhesins have been observed in ExPEC populations, such as Type 1 fimbriae, P fimbriae, curli and S fimbriae, F1C and Dr fimbriae, and afimbrial adhesins (Antão et al. 2009b; Klemm et al. 2010). In the 3770 genome we are able to observe gene loci encoding for flagella, lateral flagella, fimbriae, S fimbriae, and the putative auf fimbrial operon amongst others.

These adhesin genes suggest that 3770 could be able to adhere to a variety of sites within a host, and as discussed in Section 3.3.3, 3770 does indeed have an adhesive phenotype. The presence of a variety of fimbriae in particular suggests that 3770 would be able to adhere to a variety of host sites, as each fimbrial adhesin tends to be tropic for a particular site. Thus, a variety of fimbriae would allow adhesion to a variety of sites (Klemm et al. 2010). However, it is also possible that the presence of the K1 capsule could block many of the shorter adhesive threads. It has been shown elsewhere that presence of a polysaccharide capsule inhibits the function of short adhesins such as AIDA-I, although this does not affect longer filaments such as fimbriae (Schembri et al. 2004). A variety of shorter and longer adhesins may therefore confer the best possible compromise, allowing for expression of functional adhesins whilst capsulated, but also allowing for shorter adhesins with different tropism if in an environment where a capsule does not confer an advantage.

Possession of antimicrobial resistance genes was high, with a total of 30 genes associated with this function, as shown in Figure 16. Many of these genes encoded putative multidrug efflux pumps (Szmolka and Nagy 2013) or were associated with antimicrobial resistance based on homology to similar genes in different species. In addition, that antimicrobial resistance genes are frequently shared in the environment *via* horizontal gene transfer means that our collective knowledge of antimicrobial resistance is low (Allen et al. 2010). However, in the 3770 genome there were no well-characterised antimicrobial resistance genes. It is possible that 3770 is not under high selection pressure for

antimicrobial resistance or it is also possible that these genes are indeed linked with antimicrobial resistance but still remain to be characterised.

Toxins were observed within the 3770 genome, as shown in Figure 16. Whilst some were putative, uncharacterised toxin-antitoxin systems, 3770 also possessed *hlyF*, *vat* and *tsh*. The HlyF gene product has been recently identified as a virulence factor involved in regulating outer membrane vesicle biogenesis and subsequent release of toxins (Murase et al. 2016), however it has been associated with virulence in APEC previous to any identified function (Johnson et al. 2008b; Van der Westhuizen and Bragg 2012; Dissanayake et al. 2014). *vat* is known to be an autotransporter toxin and associated with virulence in APEC (Parreira and Gyles 2003; Pires-dos-Santos et al. 2013) however so far a substrate on which the *vat* gene product is able to act is still unknown (Dautin 2010). *tsh* encodes the temperature sensitive haemagglutinin which has recently been suggested as playing a role in the initial colonisation of the trachea of chickens (Kobayashi et al. 2010), and a separate study has also shown it to be important in the early stages of infection (Dozois et al. 2003). Possession of *hlyF*, *vat* and *tsh* provides strong evidence of 3770s ability for pathogenicity in chickens. All three have been shown to be strongly associated with virulence in chickens, with increasing evidence towards their specific roles in pathogenesis.

Possession of miscellaneous virulence factors that do not fall into other categories, such as *iss* and IbeABCT was also observed in the 3770 genome.

iss is associated with increased serum survival and significantly associated with virulence in chickens (Binns et al. 1979; Chuba and Palchaudhuri 1986; Johnson et al. 2008c). As shown in section 3.2.2, 3770 is strongly resistant to chicken serum, with little to no cell death observed after 3 hours.

The *ibeABCT* locus is associated with invasion across the blood brain barrier in NMEC (Huang et al. 1995) and has also been shown to be important for

virulence in avian species (Germon et al. 2005; Wang et al. 2011). Possession of the *ibeABCT* locus in particular could allow 3770 to cause a more systemic infection than one just purely restricted to the reproductive tract.

4.3.4 – PathogenFinder and CARD analysis

PathogenFinder (Cosentino et al. 2013) was used to analyse the entire genome of strain 3770 in order to assess potential for pathogenicity in humans. This bioinformatics tool predicted that 3770 would be pathogenic in humans with a probability of 0.932 out of a possible 1.0 indicating that pathogenicity in humans is highly likely. Given that 3770 is a field isolate of APEC and is known to be pathogenic in chickens, and also that we know of the close relationship between APEC, UPEC and NMEC (Ewers et al. 2007) this result is not surprising.

PathogenFinder also matched 701 gene sequences from pathogenic families. The majority of these sequences were matched to various *E. coli* genomes however interestingly 111 matches were to *Shigella* species. It is known that other isolates such as χ7122 are more closely related to *Shigella* species as shown in Figure 14 and elsewhere within the literature (Gordienko et al. 2013), manual curation of the 3770 genome via BLAST did not reveal high levels of similarity to *Shigella* species.

CARD (Jia et al. 2017)was used to analyse the 3770 genome for presence of antibiotic resistance genes and mutations. The analysis revealed the presence of matches to 238 antibiotic resistance genes and mutations associated with resistance to a variety of antibiotic resistance phenotypes including resistance to beta-lactams, aminoglycosides, tetracyclines, rifamycins, fluoroquinolones, and sulphonamides.

The presence of antibiotic resistance genes is unsurprising, the plasticitiy of the *E. coli* genome is high as previously discussed (Rasko et al. 2008) and antibiotic

resistance genes have been extensively studied in ExPEC populations (Heller and Smith 1973; Obeng et al. 2011; Gibreel et al. 2012; Mellata 2013).

Antibiotic resistance in APEC populations is linked with the use of antimicrobials to prevent and treat infections in poultry and to prevent subsequent economic losses. Unfortunately, the use of antimicrobials in the farming industry has been linked with antibiotic resistance in the poultry industry but also as a source of antibiotic resistance in human populations via the consumtion of contaminated meat products (Mellata 2013).

Additionally, the presence of antibiotic resistance linked genes alone does not guarantee an antibiotic-resistant phenotype. A furture avenue of work to be considered would be testing for antibiotic resistance in strain 3770 to determine which phenotypes are expressed.

4.3.5 – Presence of fragment ETT2 in 3770 genome

The upstream region containing genes *araE* to *yqeF* was observed in the 3770 genome however no further fragments were observed.

Previous work on the ETT2 gene cluster has observed that this region is not frequently observed in B2 *E. coli* and it is theorised that acquisition of this gene cluster occurred after the separation of the B2 phylogenetic group (Ren et al. 2004). 3770 belongs to the B2 phylogenetic group and as such the lack of the majority of the ETT2 gene cluster correlates with this previous observation.

5 - Population Study of SPS isolates

As previously discussed in Section 1.2, *E. coli* is a diverse pathogen with genetically distinct sub-types – broadly divided into those which infect the host gut, and those which infect outside of the host gut (ExPEC); – in which group the isolates within this study are found.

Commonly described ExPEC infections include neonatal meningitis and urinary tract infections in humans, and colibacillosis in chickens (Johnson and Russo 2002). It is theorised that bacteria are able to move from animal to human hosts *via* the food chain with strong evidence observing that strains causing UTI in women share many similarities with those isolated from local meat production animals (Jakobsen et al. 2012). This interesting finding emphasises the importance of research onto zoonoses and potential zoonoses from a human health point of view but also suggests further avenues of exploration for the evolution of pathogenic isolates within the ExPEC group. In addition, the movement of isolates between avian and mammalian hosts could suggest that ExPEC virulence factors are not host-specific. For example as previously discussed, a protein that allows a bacterium to cross the blood-brain barrier in one host may allow a bacterium to travel from the lungs into nearby organ systems in another host. Or indeed, it may also suggest that any single ExPEC isolate may happen to possess in its armoury virulence factors more typical to another subtype. The major virulence factors are summarised below.

Virulotyping by PCR enables us to distinguish between pathogenic bacteria and commensals. Multiple diagnostic panels of genes exist in the literature (Skyberg et al. 2003; Ewers et al. 2004; Schouler et al. 2012) and other industrial and clinical institutions have diagnostic panels developed in-house. These PCR-based methods of virulotyping rely on increased prevalence of target genes in the pathogenic population compared to the non-pathogenic population to ascertain whether a given strain is pathogenic. When these diagnostic tests are carried out on bacteria isolated from an infection, the test gives us an indication

as to if this infection was more opportunistic in nature (e.g. the bacteria was able to move from the gut to other tissues *via* an injury) or if the bacteria possesses factors which enable it to move from one site to another such as *via* adhesins and invasins.

There are a variety of virulence factors linked with ExPEC populations, though many can be loosely grouped into (1) those which allow the bacterium to adhere to host surfaces, (2) those which allow the bacterium to scavenge nutrients from the host environment, (3) those which allow the bacterium to evade or influence the host immune system, and (4) those which allow the bacterium to invade host cells (Johnson and Russo 2002). These will be summarised below, grouped into those more common in APEC populations followed by those more common in UPEC populations.

5.1 – APEC virulence factors

A number of ExPEC virulence factors have been hypothesised and described, for the purpose of this study a selection of the most well-defined virulence factors for APEC and UPEC populations has been chosen to ascertain whether a specific reproductive-tract-associated subpopulation of APEC has virulence factors typical to the more frequently studied colibacillosis-associated APEC populations or if a tropism towards the reproductive tract of chickens aligns with a virulence profile more alike to those in UPEC populations. These genes will be briefly described below.

5.1.1 – Increased serum survival: *iss*

Increased serum survival is highly prevalent in APEC populations, with a prevalence of over 80% usually described (Rodriguez-Siek et al. 2005a). The gene encoding for the protein is *iss* and it is usually found on the ColV/BM plasmid (Johnson and Nolan 2009).

5.1.2 – Haemolysin F: *hlyF*

A putative haemolysin has been identified in APEC populations; *hlyF*. Similarly to *iss* the gene is found on the ColV/BM plasmids, and additionally it is observed in low numbers of commensal isolates, indicating its importance for virulence in APEC populations (Johnson and Nolan 2009; Dissanayake et al. 2014). A recent study has suggested that, when expressed, HlyF was associated with increased production of outer membrane vesicles and release of toxins. This was further supported in an *in vivo* infection model of colibacillosis in which the *ΔhlyF* mutant was outcompeted by the wild-type strain, and the phenotype was regained with complementation of *hlyF* (Murase et al. 2016).

5.1.3 – Aerobactin: *iucC* and *iutA*

Aerobactin is a siderophore, allowing the bacterium to uptake iron even in environments with low levels of free iron such as serum. The aerobactin synthase is encoded for by *iucC* whilst the outer membrane protein receptor is encoded for by *iutA* (Garénaux et al. 2011).

5.1.4 – Salmochelin: *iroN*

The siderophore salmochelin is encoded for by the *iroBCEDN* locus and possession of this locus – on ColV/BM and O1 plasmids or integrated into the chromosome – is associated with virulence in APEC populations (Dobrindt et al. 2002; Johnson et al. 2006c, b; Sabri et al. 2006). *iroN* encodes for the cell-surface receptor for the siderophore machinery and chick infection experiments using a *iroN* knockout of X7122 have shown that salmochelin was important for virulence and also for persistence in deep tissues in this strain (Dozois et al. 2003).

5.1.5 – Colicin: *cva*

Colicin, encoded for by *cva*, is a plasmid-encoded virulence factor of APEC populations. Whilst it remains somewhat controversial as a virulence factor, *cva* is thought to be a useful marker for virulence and is frequently included in pathogenicity studies of APEC populations (Vidotto et al. 1990; Blanco et al. 1997; Dias da Silveira et al. 2002; Oh et al. 2011). In addition it has been hypothesised that colicins in APEC populations allow the bacteria to outcompete its competitors in the host gut (Cascales et al. 2007).

5.1.6 – Temperature sensitive haemagglutinin: *tsh*

The temperature sensitive haemagglutinin causes agglutination of chicken erythrocytes and has proteolytic activity against chicken mucins (Provence and Curtiss III 1994; Kobayashi et al. 2010). It is encoded for by *tsh* which is usually found in 50% or more of APEC populations, though prevalence appears to vary quite significantly between studies (Maurer et al. 1998; Dho-Moulin and Fairbrother 1999; Tivendale et al. 2004).

5.1.7 – Invasin: *ibeA*

A potential invasin is encoded for by *ibeA.* In NMEC populations this gene is frequently seen and is thought to play a role in allowing the bacteria to cross the blood-brain-barrier (Huang et al. 1995). Hypothesised roles for this gene product in avian populations include allowing the bacteria to survive the oxygenated environment of the avian lung, form biofilms, and invade and proliferate in host tissues. Two deletion mutant studies provide supporting evidence for the importance of this gene, with reduced virulence in an avian model (Germon et al. 2005; Wang et al. 2011).

5.2 – UPEC virulence factors

5.2.1 – P fimbriae: *papA*

The major fimbrial subunit of P fimbriae is encoded for by *papA*. P fimbriae are so named for their association with pyelonephritis and *papA* as the major subunit has been observed to be carried within APEC populations at no more than 30% (Dozois et al. 1992; Janßen et al. 2001; Johnson et al. 2001b; Delicato et al. 2003; Kemmett et al. 2014) though they are, unsurprisingly, more commonly found in UPEC populations with more than 80% of UPEC expressing P fimbriae (Tewari et al. 1994).

5.2.2 – S fimbriae: *sfaS*

S fimbriae occur in both NMEC and UPEC populations, where it is thought that they allow the bacteria to cross the blood-brain barrier or adhere to urinary tract cells (Korhonen et al. 1985, 1986). The adhesive tip of the fimbriae is encoded for by *sfaS* which typically found in UPEC populations at around 50%, NMEC populations at 24% and APEC populations at between 5-10% (Ewers et al. 2007).

5.2.3 – IrgA homologue adhesin: *iha*

The *iha* gene encodes for an adhesin that is homologous to one found in *V. cholerae* named *irgA* and was originally identified in an *E. coli* O157:H7 isolate. This protein, when expressed, has been shown to confer an adherent phenotype (Tarr et al. 2000). It has been shown to both be associated with urinary tract infections (Johnson et al. 2005b), and rarely seen in APEC isolates (Ewers et al. 2007)

5.2.4 – Cytotoxic necrotising factor 1: *cnf1*

Cytotoxic necrotising factor 1 is encoded for by *cnf1.* It causes actin reorganisation within epithelial cells and is thought to allow the bacteria to invade host cells (Fiorentini et al. 1997; Landraud et al. 2004). *cnf1* is associated with NMEC and UPEC isolates (Smith et al. 2007) but rarely with APEC isolates (Rodriguez-Siek et al. 2005b; Vandekerchove et al. 2005).

5.2.5 – Uropathogenic specific protein: *usp*

The Uropathogenic Specific Protein is encoded for by *usp* and it is thought to be homologous to the zonula occludens toxin gene found in *Vibrio cholerae.* Studies have shown it to be strongly associated with virulence in UPEC populations with correspondingly low prevalence in commensal/faecal populations (Bauer et al. 2002).

5.2.6 – Haem binding protein: *chuA*

ChuA is a haem binding protein and is therefore involved in haem transport (Torres et al. 2001), and possession of this gene is usually associated with UPEC strains. A study has shown that possession of *chuA*, alongside *vat*, *fyuA,* and *yfcV* indicates a phenotype that can efficiently colonise the urinary tract in a mouse model of ascending urinary tract infections (Spurbeck et al. 2012). Additionally, *chuA* is included in the gene panel used in the rapid PCR-based phylotyping method whereby any strain possessing *chuA* will be a member of the B2 or D phylogenetic groups and any strain lacking *chuA* will be a member of the A or B1 phylogenetic groups (Clermont et al. 2000).

5.2.7 – K1 capsule: *kps MT k1*

The *E. coli* K1 capsule is a polysialic acid capsule. It is encoded by a gene cluster that includes the gene *kps MT k1.* The K1 capsule is strongly associated with human extra-intestinal infections, both ascending urinary tract infection and neonatal meningitis (Bliss and Silver 1996) and has been identified as a virulence factor important for serum resistance and protection from phagocytic killing (Kim et al. 2003).

5.2.8 – α-haemolysin: *hlyD*

The α-haemolysin is a type 1 secreted protein encoded for by the *hlyABCD* operon. HlyD is a membrane fusion protein and interacts with TolC. The α-haemolysin is a pore-forming toxin which is able to target epithelial cells and macrophages, suggesting a dual purpose in both enhancing bacterial translocation within the urinary tract through the shedding of epithelial cells but also attenuation of phagocytosis allowing the bacteria to evade the host immune response as summarised by (Dhakal and Mulvey 2012).

The α-haemolysin is infrequently found in avian ExPEC isolates (Rodriguez-Siek et al. 2005a).

5.3 –Population studies

There have been a number of previous population studies carried out on ExPEC populations focusing on single ExPEC subtypes or comparing across subtypes. Despite a rather large volume of evidence, it is difficult to ascertain the importance of specific genes for virulence in specific populations as whilst one study may show evidence suggesting a genes importance in a certain population another may see it occurring rarely in their study population.

An important study by Ewers et al. compared 526 isolates originating in human and animal infections; neonatal meningitis, UTI and avian colibacillosis. They observed little variation in the frequency that most chromosomally encoded genes were carried equally in all three populations (with exceptions being *iha, afa/draB, sfa/foc* and *hlyA).* However there were significant differences in the carriage rate of plasmid encoded genes (especially the ColV/BM plasmids) and also, interestingly, that invasion-associated genes were more frequently observed in NMEC and APEC strains compared to UPEC strains (Ewers et al. 2007).

A study by Kemmett et al. took a longitudinal approach exploring the carriage of virulence associated genes in commercial broiler flocks in the UK and the virulence factors carried by strains isolated from diseased chickens within those same flocks. In this study, 324 isolates from diseased chickens were studied and a high degree of strain diversity was noted. Genes that were significantly associated with strains isolated from diseased chickens were *irp2*, *papC*, *iucD*, *cvi*, *sitA* and *ibeA*. Genes associated with iron acquisition were also noted to be present in high numbers within this population (Kemmett et al. 2013).

Most recently, a study by Olsen et al. characterised 100 isolates from diseased birds within layer flocks in Denmark. The diseased birds all had lesions characteristic of salpingitis-peritonitis-salpingoperitonitis syndrome. The most frequent virulence factor was *iroN,* which was isolated from 100% of the isolates, and the most infrequent virulence factor was *astA* seen in 25% of the isolates. Also observed in over 70% of this study population were *iss, ompT, papA, iucD, vat* and *cva* (Olsen et al. 2016).

For the purpose of this study, 188 isolates associated with salpingitis-peritonitis-salpingoperitonitis were each assayed for the presence or absence of 16 ExPEC virulence factors; 8 of which are more commonly associated with APEC and 8 of which are more commonly associated with UPEC.

5.4 – Results

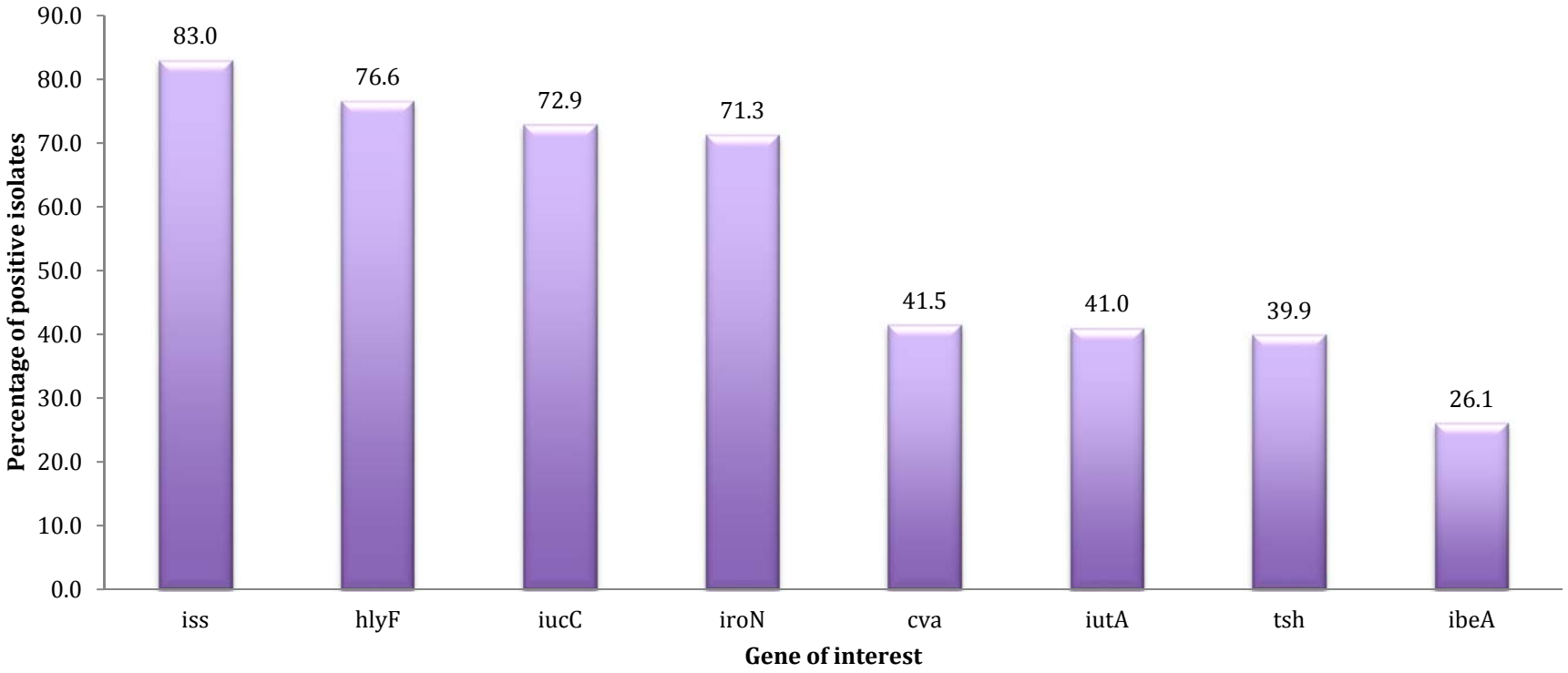

Figure 19: Prevalence of carriage of genes associated with APEC pathotypes.

DNA extracted from SPS-associated *E. coli* isolates underwent PCR reactions to ascertain the presence or absence of APEC virulence genes.

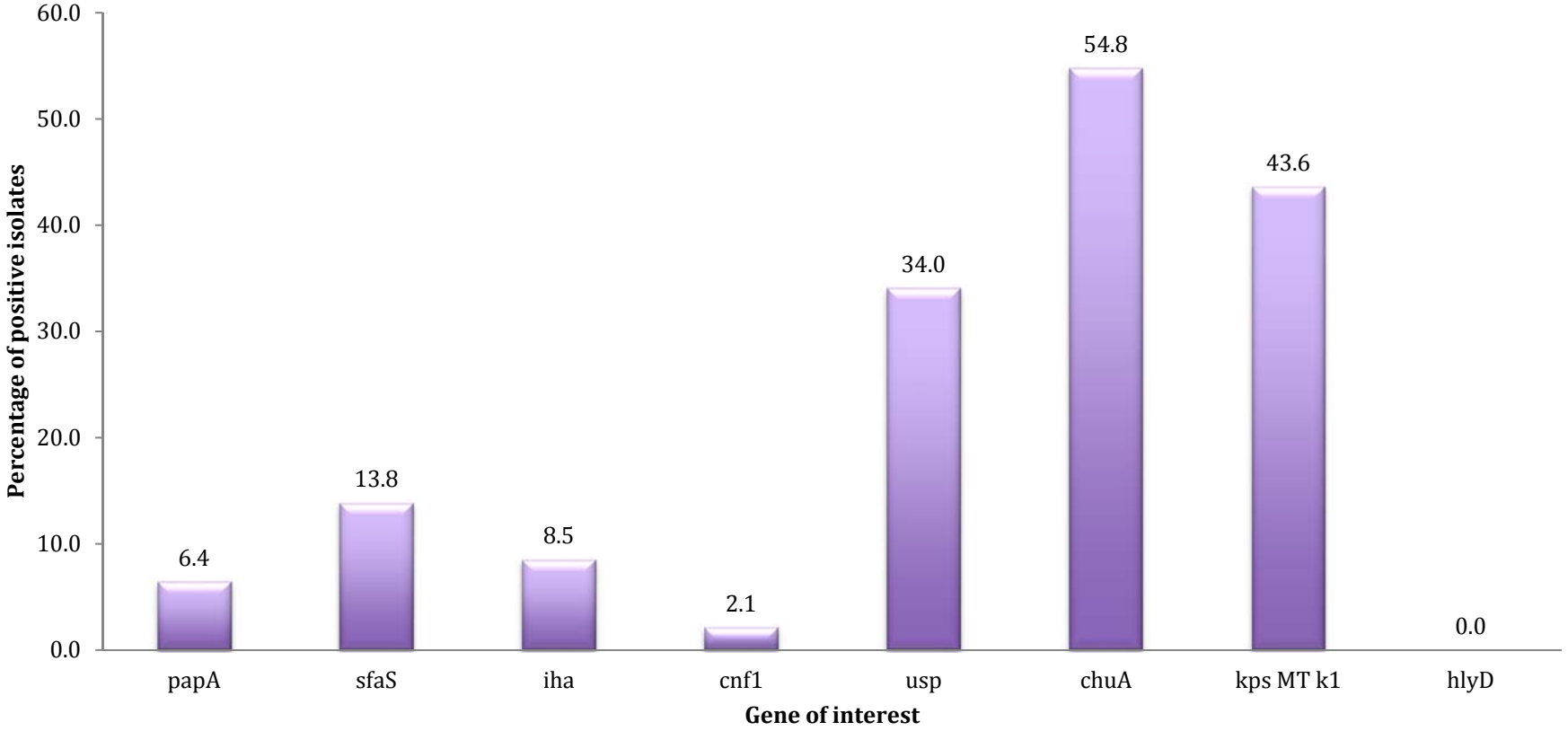

Figure 20: Prevalence of carriage of genes associated with UPEC pathotypes.

DNA extracted from SPS-associated *E. coli* isolates underwent PCR reactions to ascertain the presence or absence of UPEC virulence genes

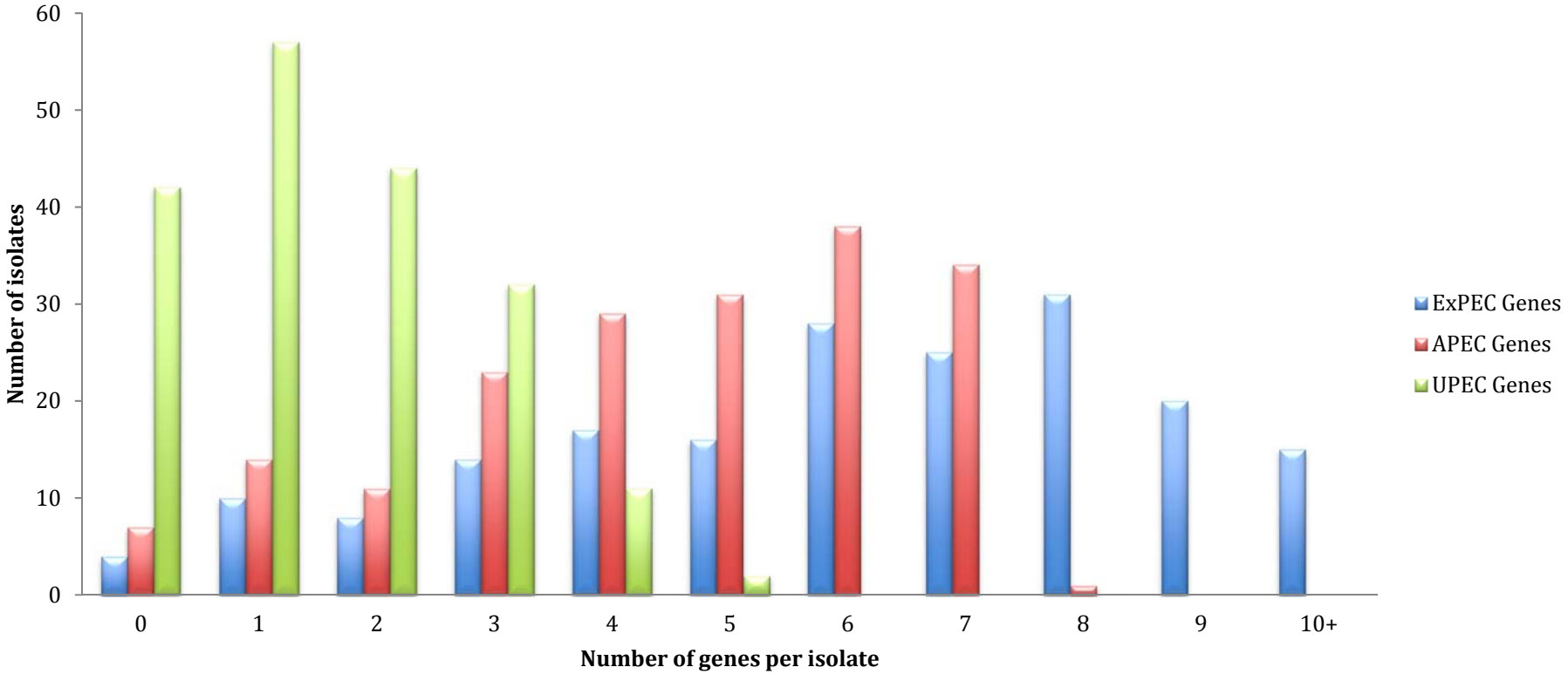

Figure 21: Number of genes carried per isolate, overall and by subtype.

DNA extracted from SPS-associated *E. coli* isolates underwent PCR reactions to ascertain the presence or absence of APEC and UPEC virulence genes.

N positive genes per isolate	N total isolates	N unique profiles
12	1	1
11	6	2
10	8	7
9	20	15
8	31	25
7	25	22
6	28	22
5	16	13
4	17	12
3	14	9
2	8	8
1	10	5
0	4	1
-	188	142

Table 4: Number of unique profiles in population study

5.4.1 – Prevalence of APEC associated virulence factors was high

In this study population, carriage of genes usually associated with APEC pathotypes was high, with carriage rates of at least 26% (*ibeA*). 3 out of 8 genes were found in around 40% of the study population: *cva* (41.5%), *iutA* (41%) and *tsh* (39.9%). The remaining 4 genes of the panel were found in over 70% of the isolates, these being *iroN* (71.3%), *iucC* (72.9%), *hlyF* (76.6%) and *iss* (83%).

5.4.2- Prevalence of UPEC associated virulence factors was varied

Within this study population, carriage of genes usually associated with UPEC pathotypes was varied. The three most frequently observed genes were *chuA* (54.8%), *kps MT K1* (43.6%) and *usp* (34%). Observed infrequently were *sfaS* (13.8%), *iha* (8.5%), *papA* (6.4%) and *cnf1* (2.1%). *hlyD* was not observed within this study population.

5.4.3 – Number of genes carried per isolate was varied

Within this study population, the number of these ExPEC genes carried by each isolate was highly variable. 4 isolates possessed none of the genes tested for. The majority of isolates tested (166) carried 3+ virulence genes. 66 isolates possessed 8+ of these virulence genes.

Carriage of APEC associated virulence factors was higher in this study population than carriage of UPEC associated virulence factors as shown in Figure 21. 104 isolates carried 5+ APEC virulence factors compared to 2 isolates carrying 5+ UPEC virulence factors. 7 isolates carried 0 APEC associated virulence factors, contrasting with 42 isolates carrying 0 UPEC associated virulence factors.

There were 142 different virulence profiles observed within the population, no more than 3 isolates shared an identical profile.

5.4.4 – Prevalence of iron uptake genes was high

The prevalence of iron uptake genes was high within this study population, with *iucC* and *iroN* present in over 70% of isolates, *chuA* in 54.8% of isolates and *iutA* in 41% of isolates.

5.4.5 – Prevalence of adhesion and invasion related genes was low

Within this study population the prevalence of adhesins and invasins was low. The most frequently observed adhesin or invasin was *ibeA*, (26.1%) followed by *sfaS* (13.8%). *iha* (8.5%) and *papA* (6.4%) were also infrequently observed in these isolates.

5.4.6 – Prevalence of toxins was varied

In this population the prevalence of toxin encoding genes was highly varied. The putative avian haemolysin *hlyF* (76.6%) was the most frequently observed gene within this category. Three toxin-encoding genes were observed in 30-40% of the study population: *cva* (41.5%), *tsh* (39.9%) and *usp* (34%). Infrequently observed were *cnf1* (2.1%) and *hlyD* (0%)

5.5 – Discussion

This study investigated the prevalence of a panel of virulence factors of which half were more commonly associated with APEC pathotypes and half more commonly associated with UPEC pathotypes. The primary aim was to investigate if a specific SPS-associated population of APEC isolates had virulence factors typical of the more typical colibacillosis-associated APEC pathotype or if typical UPEC virulence factors might be more frequently found in this population. Additionally, this study aimed to characterise the virulence factors prevalent in this SPS-associated population isolated from layer flocks throughout the UK. Previous population studies consistently showed high levels of variation within the ExPEC pathotype itself and also within the particular sub-pathotypes. Variation also exists between studies, showing that virulence is a complex matter not yet fully understood.

Consistent with previous studies, a high level of variation was seen in this study also (Rodriguez-Siek et al. 2005a; Ewers et al. 2007; Kemmett et al. 2013; Olsen et al. 2016). Overall, possession of typical APEC virulence factors was high, with 104 of 188 isolates carrying 5+ of the 8 virulence genes tested for. Possession of UPEC associated virulence factors was comparatively lower, with only 2 isolates possessing 5+ of the 8 virulence genes tested for, and 42 carrying 0 UPEC associated virulence factors compared to 7 possessing 0 APEC associated virulence factors.

142 different profiles were observed within a population of 188 samples with no more than 3 isolates sharing an identical profile. This data further shows that overall variation within this population of SPS isolates is high.

Additionally, two isolates did not possess any virulence factors from either type. This data is consistent with a previous study within this lab whereby some strains isolated from lesions within diseased birds do not possess any of the virulence factors tested for (Kemmett et al. 2013). Of the 7 isolates containing 0

APEC associated virulence genes, 5 did contain UPEC associated virulence factors, which could have proved advantageous when causing infection in the host. Of the 2 isolates containing 9 ExPEC virulence factors, as *E. coli* is known to be an opportunistic pathogen, this could reflect on the health of the host rather than the ability of the particular isolate to cause infection.

As expected, prevalence of typical APEC virulence factors was high. The most frequently observed virulence factor was *iss* with a prevalence of 83% within this population, which is consistent with other studies showing this gene to be highly prevalent in their populations (Rodriguez-Siek et al. 2005a; Ewers et al. 2007; Olsen et al. 2016). Also observed frequently in this population was the putative avian haemolysin *hlyF* observed in 76.6% of isolates. This gene product has been recently characterised as a factor involved in outer membrane vesicle synthesis (Murase et al. 2016) and has been suggested as a gene which is highly indicative of APEC pathogenicity (Dissanayake et al. 2014). Interestingly the gene encoding for HlyF is found on the ColBM plasmid, which is itself strongly linked with APEC pathogenicity (Johnson et al. 2006b).

Prevalence of UPEC-associated virulence factors was varied, though overall there was less frequent carriage of UPEC virulence factors than APEC virulence factors. The most frequent UPEC virulence factors were *chuA* which is involved in iron acquisition, part of the K1 capsule gene cluster *kps MT K1* and *usp* which is a putative toxin. Whilst *chuA* is more strongly associated with UPEC isolates, this data is consistent with other studies which have observed it within their study populations though not in high frequencies (Matter et al. 2011). As iron uptake systems are of high importance for ExPEC bacteria it has been noted that isolates often possess multiple iron acquisition systems (Garénaux et al. 2011).

Also of interest, is that *chuA* is part of the panel of genes used in the rapid PCR-based phylotyping method. Samples positive for *chuA* will belong to either phylogenetic group B2 or D. That this study population observed *chuA* in just

over half of the isolates (54.8%) we can assume that many isolates within this population belong to phylogenetic groups A and B1. Most ExPEC isolates are typically found in the phylogenetic group B2 whereas A and B1 are more frequently associated with environmental isolates and also those isolated from fish, frogs and reptiles (Gordon et al. 2008). This data could be concordant with that seen in Ewers et al, in which 46.1% of the ExPEC study population was observed to be phylogenetic group A (Ewers et al. 2007).

The K1 capsule encoding gene *kps MT K1* was observed in 43.6% of this study population. This is notably higher than the prevalence observed in other studies (Rodriguez-Siek et al. 2005b; Ewers et al. 2007), potentially indicating a role for the K1 capsule in infections of the reproductive tract.

Iron acquisition systems were the most frequently category of gene observed, with both *iucC* and *iroN* seen in >70% of isolates. As mentioned previously, chuA was also frequently observed, and the lowest iron-uptake associated gene was *iutA* seen in 41% of isolates. This data is concordant with other published studies, in which possession of iron uptake systems is high within ExPEC populations (Ewers et al. 2007; Garénaux et al. 2011; Kemmett et al. 2013; Olsen et al. 2016). Iron uptake systems are of particular importance for bacteria that reside within a host environment; as a mechanism to prevent bacterial growth animals sequester free iron tightly into metalloprotein complexes such as haem and ferritin to create an environment unfavourable to bacterial growth. Bacteria which are able to obtain and use this iron are therefore at a competitive advantage against those bacteria that lack this ability (Garénaux et al. 2011), however a level of functional redundancy must also be considered. In particular, enterobactin has previously been shown inessential for virulence if other iron-uptake systems are present (Caza et al. 2011).

Genes associated with adhesion and invasion were seen infrequently in this study. *ibeA* was observed in 26.1% of the study population. This data is strikingly

similar with that seen Ewers et al, in which the frequency of *ibeA* in APEC populations was reported to be 26.2% (Ewers et al. 2007), however this contrasts with the lower prevalence of *ibeA* observed by Kemmett et al. (Kemmett et al. 2013). The population studied in both this study and Ewers et al. are entirely or mostly derived from reproductive tract infections. In contrast, the population studied in Kemmett et al. was derived entirely from broilers. It is possible that the different prevalence of *ibeA* in these studies indicates that *ibeA* plays a more important role in reproductive tract infections of the mature hen than in colibacillosis in broilers

Other UPEC associated adhesin and invasion associated genes such as *sfaS* and *iha* were observed infrequently in this population, which is also consistent with that seen in Ewers et al, though interestingly the prevalence of the papC gene was notably lower in this study than in other populations (Ewers et al. 2007). There are many adhesins found in ExPEC populations (Antão et al. 2009b), thus it is possible that these isolates do have an adhesive phenotype conferred by an adhesion not tested for in this study.

Toxin production gene prevalence was variable within this population. The most frequently observed toxin-associated gene within this study was *hlyF*, as discussed earlier in this section. Observed at a moderate frequency of between 30-40% were *cva, tsh* and *usp*. Other studies have observed *cva* at higher frequencies such as 72.3% as seen in Ewers et al, which conflicts with the data seen within this study. Although as a potential role for colicin in APEC infections has, as yet, not been identified, more information is required to confidently state its importance in these populations. Within this study the prevalence of *tsh* was observed at 39.9% which is slightly lower than the frequencies seen in other population studies but not significantly so (Rodriguez-Siek et al. 2005b; Ewers et al. 2007; Olsen et al. 2016).

usp is not well-studied within APEC populations, however it has been identified previously (Kariyawasam et al. 2007), thus it appears that this is the first study to analyse the frequency of this gene in a large population of APEC isolates; further study is most certainly required for this specific gene.

Finally, observed at a low frequency (*cnf1*; 2.1%, *hlyD*: 0%) were two UPEC associated virulence factors, whereby the frequencies observed within this study are consistent with the frequencies seen in other studies (Rodriguez-Siek et al. 2005b; Ewers et al. 2007) agreeing that these genes are indeed strongly associated with UPEC isolates. Given the presence of other toxin genes within these populations, it could be considered that toxins might be more host-specific than other virulence factors.

Overall, this study has observed varied frequency of the virulence genes investigated, which is consistent with the flexible nature of *E. coli* as a pathogen and its diverse genomic composition. Overall, possession of ExPEC virulence – associated genes was high. Unsurprisingly, APEC associated genes were higher in this APEC population than UPEC associated genes, although some UPEC associated genes such as *chuA* were observed in relatively high frequencies.

Also consistent with the literature was the high prevalence of iron uptake systems; this category of virulence factor clearly highly important for ExPEC pathogenesis. Adhesion and invasion genes were observed in lower frequencies than expected, however not all potential adhesion and invasion genes were investigated thus it is entirely possible that this population might still have adhesive and/or invasive phenotypes; but that these could be conferred by alternative virulence factors. Finally, toxin associated genes were present within this population, most frequently observed was *hlyF* however *tsh* and *cva* were also found at a moderate frequency within this population.

6 – General Discussion

The main findings presented in this thesis are as follows.

Strain 3770 showed characteristic growth characteristics, was able to withstand the bactericidal effects of serum, adhere to epithelial cells and persist short-term within heterophils. 3770 was shown to be able to induce a reproductive tract infection in previously healthy laying hens, when administered *via* the aerosol route.

Genome sequencing of strain 3770 revealed a 5.02Mb genome without detectable plasmids. A high degree of concordance to other ExPEC was observed, however phylogenetic methods revealed that the closest sequenced relative to 3770 is the adherent-invasive *E. coli* LF82. The sequence type (MLST type) of 3770 was shown to be ST141. Virulence genes were frequently found within the genome of 3770, with high numbers of genes associated with iron uptake, antimicrobial resistance, cell surface factors and toxins observed.

A wider population study of 188 SPS isolates revealed a high degree of variation between isolates, with overall high prevalence of APEC-associated virulence factors and a lower, more varied prevalence of UPEC-associated virulence factors within this population.

APEC are an important pathogen within the poultry industry, with a high prevalence of disease in both laying hens and broiler birds (Yogaratnam 1995; Cumming 2001). Isolates within this pathotype are capable of causing a variety of infections, most commonly colibacillosis, colisepticaemia and cellulitis in broiler birds (Yogaratnam 1995; Elfadil et al. 2014) and infections of the reproductive tract in laying hens (Cumming 2001; Vandekerchove et al. 2004).

Furthermore, it is highly likely that the source of many APEC infections is the intestinal tract of the chicken itself, with studies showing potentially pathogenic

E. coli within the gut population of apparently healthy birds (Kemmett et al. 2013) – suggesting that a complex relationship between the host health status and the virulence profile of the bacterium plays a very large role in the development of infection (Collingwood et al. 2014).

Cell-based characterisation methods revealed that 3770 displays a similar phenotype to APEC O1, UTI89 and χ7122, which were used as comparator ExPEC isolates within this study. All four isolates were able to withstand the bactericidal effect of serum, adhere to epithelial cells *in vitro*, and persist for a short period of time in heterophils. These abilities are well-characterised within APEC populations and are considered essential for virulence (Pourbakhsh et al. 1997a; La Ragione et al. 2000a; Nolan et al. 2003; Ramirez et al. 2009; Antão et al. 2009b).

The ability of 3770 to cause reproductive tract infections *via* the aerosol route in previously healthy laying hens was particularly interesting. Previous studies have shown successful infections caused *via* this route when the birds were exposed to nebulized, aerosolised bacteria whilst within an isolator (Landman et al. 2013) however this highly contained model does not reflect real world conditions within a hen house. This study showed that infections of the reproductive tract could be induced *via* inhalation of bacteria under otherwise normal conditions, and as such shows that this route of infection is highly likely to take place outside of experimental settings.

Furthermore, this data shows that 3770 is able to cause infections within the reproductive tract without signs of additional airsac colonisation, indicating that 3770 was able to leave the airsac environment soon after entry, potentially *via* the use of the *ibeABCT* encoded invasin (Wang et al. 2011, 2012). The lack of observed inflammation within the airsacs indicate that the bacteria did not leave the airsac environment through damaged lesions caused by an influx of heterophils as suggested previously (Dziva and Stevens 2008).

The 3770 genome was characteristic of *E. coli* genomes, both in size and G+C content. There were large regions of homology with APEC O1 and UTI89, but less so when compared with χ7122. This is perhaps unsurprising as χ7122 has been shown to have evolved from a different route to conventional ExPEC (Dziva et al. 2013).

Interestingly the sequence type of 3770 has been observed before in populations associated with reproductive tract infections (Olsen et al. 2011; Pires-dos-Santos et al. 2013). As reproductive tract associated are relatively understudied and given the lack of epidemiological data on the common sequence types within this population, it is possible that ST141 is not an uncommon sequence type for reproductive tract associated isolates. More work is required to investigate this hypothesis.

The genome of 3770 revealed high levels of virulence gene carriage, including all four siderophore gene loci carried within ExPEC populations (Garénaux et al. 2011). While a level of functional redundancy has been suggested for iron uptake systems within ExPEC (Garcia et al. 2011), many studies have shown the importance of iron uptake systems for virulence within these populations (Sabri et al. 2006; Feldmann et al. 2007; Holden et al. 2012; Xiong et al. 2012; Gao et al. 2012). Iron uptake systems are vital for bacteria; within a host environment free iron is tightly sequestered into proteins such as haem, thus any bacteria in a host environment must be able to use specific iron uptake mechanisms in order to replicate (Garénaux et al. 2011).

Additionally, iron uptake mechanisms are linked to resistance to oxidative stress (Sabri et al. 2006), which would also be an advantage within a host environment. Possession of the full K1 capsule gene cluster was also observed within the 3770 genome. The *kpsMT* genes had been observed during the initial characterisation

of the isolate (Timothy et al. 2008), however possession of the full 14 gene locus was by no means guaranteed.

It is particularly interesting to consider the potential link between the genome content and the phenotype observed in the cell-based studies and chicken infection. Little is known about the specific immune response to *E. coli* within the avian reproductive tract however it is well known that large numbers of lymphocytes and macrophages within the oviduct and ovarian follicles, as are antimicrobial peptides (Wigley et al. 2014). Perhaps the high level of genes associated with protection, evasion, or modulation of the host immune system such as *iss,* the K1 capsule cluster, and iron acquisition systems could confer an advantage in the avian reproductive tract? Or perhaps the presence of the *ibeABCT* locus allows for rapid translocation from the airsac to the reproductive tract? A potential route to elucidate the links between virulence content and phenotype would be a combination of transcriptomic studies and signature-tagged mutagenesis, which would allow us to see which genes are switched on during infection and also which genes are essential for virulence for strain 3770.

In Chapter 5, virulence gene carriage within an SPS-associated population of APEC was revealed to be highly variable. Carriage of virulence genes associated with APEC was higher than carriage of UPEC associated virulence genes, however the results were not concordant with the literature. In particular, carriage of the K1 capsule genes was significantly higher in this population than in other studies (Rodriguez-Siek et al. 2005b; Ewers et al. 2007), suggesting that perhaps the K1 capsules – or indeed any capsule - could confer an advantage within the reproductive tract environment. As capsules are strongly linked with evasion of the host immune system (Roberts 1996), it would be of interest to determine at which point in the infection process capsules are expressed, as possession of the K1 capsule is also thought to be involved in translocation of

the blood-brain-barrier (Kim et al. 2003), and thus potentially could be involved in translocation within the avian host also.

Another facet of this experiment in which the data obtained conflicted with that seen in other studies was the presence of *ibeA.* In this study the prevalence was 26.1%, which is strikingly similar to that seen in Ewers et al, but is higher than the prevalence seen by Kemmett et al. (Ewers et al. 2007; Kemmett et al. 2013). In this instance, it is possible that the study population is a large factor in this observation. In this study, and in the study performed by Ewers et al. the study population is entirely, or largely laying hens. In the study performed by Kemmett, the study population is entirely broiler birds. This suggests that *ibeA* is more important in virulence of reproductive tract infections in layers than in the colibacillosis syndrome typically observed in immature broiler birds.

Whilst overall 3770 was revealed to be characteristic of ExPEC in cell-based experiments, and also characteristic in its virulence gene profile, differences were observed both in its ability to cause infection in the reproductive tract and also within the phylogenetic analysis.

3770 was more capable of causing reproductive tract infections than χ7122, which in turn appeared slightly more capable of causing systemic infections. Here we see variable pathotypes within an APEC population, providing evidence to suggest that there is not one single APEC pathotype but rather a variety, each associated with a different disease syndrome (Collingwood et al. 2014).

Additionally, phylogenetic analysis of 3770 was performed using whole-genome alignments to create a phylogenetic tree, which revealed greater similarity to an adherent-invasive isolate LF82 (Miquel et al. 2010) than to APEC O1 or UTI89. It would not be novel for an APEC isolate to originate from an enteropathogenic isolate of *E. coli*. Previous studies on strain χ7122 has shown that the strain originates from enterotoxigenic *E.* coli (Dziva et al. 2013) and is therefore is

more closely related to enterotoxigenic *E. coli* isolates and *Shigella* than to any other member of the ExPEC family.

Additionally, it has been observed that whilst most ExPEC are derived from limited clonal pools, the flexibility of the *E. coli* genome allows for ExPEC isolates to occur sporadically from other lineages (Johnson et al. 2006a). A criticism of these methods would be that recombination events are too frequent within *E. coli* populations, and if spontaneous recombination allows for ExPEC isolates to occur within other lineages, evolution of APEC begins appear increasingly random.

While this study gave some insight into the phenotype and genotype of strain 3770, the close genetic relationship between 3770 and LF82 in particular raises many questions that could be further explored. It would be interesting and of potential value to compare these genetically similar isolates for their ability to interact with human epithelial cell lines such as M cells and potentially a murine ileal loop model of Crohn's Disease such as that seen in (Chassaing et al. 2011). As it has been repeatedly observed that UPEC strains and APEC strains share a lot of similarities CITE and that human UTIs can originate from APEC bacteria on food such as poultry products (Jakobsen et al. 2010, 2012; Bergeron et al. 2012; Manges and Johnson 2012), this line of research could suggest that the zoonotic potential of 3770 may not be limited to UTI alone.

This study provides valuable information on an under-studied subtype of APEC infection, which is a large problem within the poultry industry. Furthering our understanding of the pathogenesis of these isolates is an important task in order to provide better disease control measures.

Our search for a specific pathotype for APEC perhaps could be reframed as a search for greater understanding of the various pathotypes within APEC populations. As we learn more and more about the complex relationships

between the bacteria and the host, and the complex relationships between the various subtypes of *E. coli* – the assumption that isolates causing infections of the airsacs and those causing infections of the reproductive tract belong to a single 'APEC pathotype' begins to seem a little misguided and oversimplified.

Appendix I – Collingwood *et al*

frontiers in
VETERINARY SCIENCE

PERSPECTIVE ARTICLE
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Is the concept of avian pathogenic *Escherichia coli* as a single pathotype fundamentally flawed?

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Avian Pathogenic *Escherichia coli* (APEC) is a major pathogen within the poultry industry. However disease, especially in broiler chickens, may be caused by range of *E. coli* genotypes that carry few, if any, virulence factors associated with APEC. Furthermore, commensal *E. coli* in the intestines of healthy birds may carry an array of APEC virulence factors suggesting they have potential to cause disease when opportunity arises. Given the diseases caused by APEC, namely colibacillosis and salpingitis peritonitis syndrome, are syndromic in nature and the great diversity of the strains causing disease we suggest it is wrong to consider disease as the result of a single APEC pathotype. Whilst it is clear certain pathogenic *E. coli* can be considered as APEC, much of the disease-associated with *E. coli* in domestic poultry is as much a consequence of increased host susceptibility due to stress, immunosuppression, co-infection, or poor welfare. This leads to more "opportunistic" infections rather than the result of infection with a specific pathotype. As such the current use of the term APEC for all cases of *E. coli* infection in the chicken is fundamentally flawed.

Keywords: chicken, *Escherichia coli*, APEC, broiler chicken, eggs

Avian Pathogenic *Escherichia coli* (APEC) is both a primary and secondary pathogen of the chicken and other avian species (1). It is considered to be a member of the extra-intestinal pathogenic *E. coli* (ExPEC) along with human Uropathogenic (UPEC) and neonatal meningitis-associated *E. coli* (NMEC) that cause disease outside the intestine. APEC infection may occur in broiler (meat) chicken, turkey, and egg-laying sectors. In all sectors, infection is syndromic in nature. In the broiler chicken, APEC infections are considered to typically lead to colibacillosis; a syndrome that includes respiratory tract infection, air sacculitis, pericarditis, perihepatitis, splenomegaly, and swollen head syndrome. In mature laying hens, reproductive tract infection leading to salpingitis or salpingo-peritonitis syndrome (SPS) is common.

Avian pathogenic *Escherichia coli* is amongst the greatest health threats to the developed poultry industries and its emergence perhaps reflects the decrease in the prophylactic use of antimicrobials or their use as growth promoting agents. Furthermore, the close genetic relationship between APEC and other ExPEC associated with human disease along with evidence from experimental animal models have lead to suggestions that APEC may represent a zoonotic risk (2–8).

Whilst in recent years, APEC has become accepted as a primary pathogen rather than a consequence of respiratory or immunosuppressive viral infections, our understanding of APEC and its pathogenesis has remained relatively limited, due, at least in part, to its great diversity and genomic plasticity (3, 4, 9–13). This variation is true of other ExPECs too (14). *E. coli* associated with human intestinal disease harbor certain defining virulence factors such as Type III secretion systems, Shiga-like toxins, and enterotoxins. ExPEC are, in general, less well defined in terms of their virulence determinants and are more variable between isolates. These virulence determinants include bacterial capsule that helps avoid host innate immunity, adhesins such as fimbriae involved in attachment to host cells and tissues, hemolysins that lyse host blood cells and multiple systems for acquisition of iron needed by the bacterium. In APEC, no single common virulence factor has been identified in all strains. Although certain genes associated with pathogenicity are common in APEC, including: *iss* associated with serum resistance (15), *ibeA* associated with invasion (16), and *sitA* associated with iron acquisition, they are not found in all isolates. Virulence genes including *iss*, *iroN*, and the *iuc* and *cva* operons are often associated with large plasmids including the Colicin V (ColV) plasmid (11). Certain *E. coli* serotypes such as O1 and O78 are more frequently associated with colibacillosis. Potentially these lineages harbor the genetic backbone required to acquire virulence mechanisms. Representatives of these serotypes have been genome sequenced and characterized (17, 18) and whilst there is little doubt these represent highly pathogenic variants of APEC, the reality is that in commercial broiler chickens colibacillosis is caused by a wide range of *E. coli* serotypes. Recently in our laboratory we have, for the first time, compared *E. coli* isolates causing colibacillosis within a broiler flock with those carried as intestinal commensals (19). These data showed that many colibacillosis – associated isolates carry few, if any, of the genes most commonly ascribed as APEC virulence factors. In essence, they have the genotype of "intestinal commensals." Equally, virulence genes may be found in "commensal" *E. coli* residing in the intestines of presumed healthy birds. Furthermore, isolates from cases of colibacillosis are not associated with any specific phylogenetic group. It appears that soon after

hatch chicks acquire a diverse range of *E. coli* as part of their microbiota, likely to be sourced from the hatchery environment. Within this population are isolates carrying virulence-associated genes (1), though the frequency of such genes decreases as birds age (19). *E. coli* found in the intestinal tract are likely to form a reservoir of potential infection, we have previously termed such isolates as potential APEC or pAPEC (19), and may be associated with early infection and mortality. Such isolates could be considered opportunists in a compromised avian host as a consequence of production-related stress, immunosuppression, or prior infection (1, 19, 20). Whilst opportunistic infections are likely to reduce in likelihood in older birds, they represent a clear risk in broiler production.

In laying hens, the situation is a little clearer. We have previously shown that APEC isolates from an outbreak of SPS in a commercial layer flock was caused by a single isolate that displayed features of both APEC and UPEC (2). Full genomic analysis of this isolate is ongoing. Recently, we have looked at the distribution of key virulence in genes in 188 SPS isolates from the UK through PCR. These findings suggest the majority possess *iss*, *hlyF* (a hemolysin), and the iron acquisition genes *iucC* and *iroN* but other virulence genes are less frequently found (**Figure 1**). Whilst within the 188 isolates there is variation, there does at least seem to be at least greater commonality of virulence factors than in colibacillosis, although seven of these isolates do not posses any of the genes screened for. This closer relationship between certain genotypes and disease and layers has also been described in Denmark where it appears certain lineages are common throughout the country, though there is still considerable diversity (21). Intriguingly in our UK-based studies although genes of the *iuc* aerobactin operon are found commonly (over 70%) in both SPS isolates and in systemically isolated *E. coli* in colibacillosis, *iss* which was found in 83% SPS isolates was only found in 25% of broiler colibacillosis isolates whilst *ibe*, was found in more than 60% of broiler isolates but was found in <25% of layer SPS isolates. This may be coincidence, but does suggest that different virulence factors may play roles causing what are very different diseases. The use of the subcutaneous infection model for cellulitis in day old chicks has recently identified *pic*, a serine protease, as a putative a virulence factor (22). However as yet, this is only an association with reduced virulence, has no mechanism ascribed and has not been identified in other models. A problem is that phenotypes of virulence factors in the chicken are poorly understood, partly as a consequence of the difficulty in reproducing experimental infections, in particular of the reproductive tract, so making our understanding of the mechanisms of APEC disease rather rudimentary. Recently developed infection models for SPS may clarify some of the virulence factors and mechanisms that underlie this disease syndrome (23, 24). However, such models rely on direct delivery to the reproductive tract and, in common with colibacillosis models, where delivery is direct to the air sacs (18, 25), may fail to detect important factors involved in the initiation of infection and colonization of tissues.

So one may pose the question should APEC be defined as any *E. coli* isolated from a diseased or sick bird or should the term be more narrowly defined? It is clear that certain well-characterized isolates can be defined as APEC. They can cause disease in experimental models and possess a range of virulence factors. However disease, and in particular colibacillosis, may result from infection with an isolate which bears few or none of the hallmarks by which we would define APEC, other than the fact it has caused disease. The

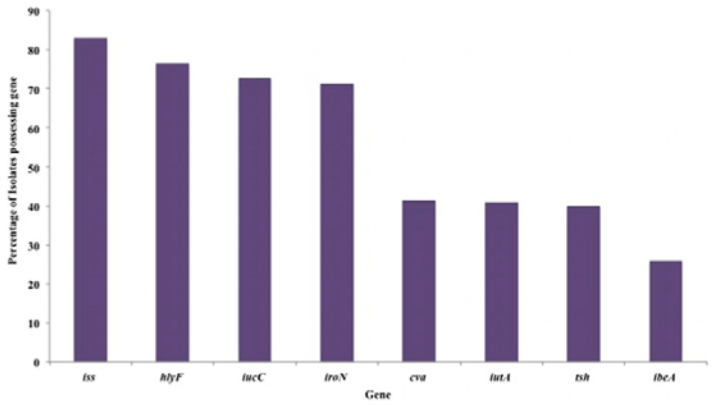

FIGURE 1 | Distribution of APEC associated virulence determinants in 188 *E. coli* isolates from cases of salpingitis peritonitis syndrome in UK laying hens. Virulence determinants detected by PCR using previously described methods (13, 19). The genes tested and their function were *iss* (increased serum survival), *hlyF* (a hemolysin), *iucC* (aerobactin), *iroN* (iron acquisition), *cva* (colicin operon), *iutA* (iron transport), *tsh* (a hemagglutinin), and *ibeA* (invasion). Data previously presented at American Society for Microbiology, Annual General Meeting, Denver, June 2013.

use of the term APEC for *E. coli* that cause what in all likelihood is opportunistic infection in the chicken is fundamentally flawed. It is perhaps more appropriate to consider both colibacillosis and SPS as disease syndromes caused by an array of variable genotypes, that are as dependent on host susceptibility as any virulence factor possessed by the pathogen. It is probably fair to say APEC is successful pathogens that are far more likely to lead to disease in poultry than commensal *E. coli*. However, disease is not just restricted to those isolates we can define as having an APEC pathotype, and trying to pin all incidences of colibacillosis or SPS on an APEC pathotype is flawed. Of course such concepts are more challenging both to scientists and producers when identifying specific causative agents and their mechanisms are the norm.

There are also implications in disease control. It is difficult to control bacteria that are normally a commensal and although vaccination against APEC (26), especially in laying hens, has value it is not feasible to achieve either economically or immunologically in young broiler chicks. As such, good hatchery hygiene and management are important in controlling early mortalities, with good management and welfare likely to reduce the risk of colibacillosis in growing broilers. This also includes effective control of other pathogens including respiratory viruses where *E. coli* is a common secondary pathogen. Vaccination may not be effective against such a diverse microbial population and removal of *E. coli* as part of the microbiota may have other implications we cannot foresee. That said, approaches to sequence multiple genomes of *E. coli* from the chicken could reveal a common "core" genome in disease-associated *E. coli*, identifying a genetic relationship that cannot be found when considering virulence factors alone. Such an approach may also lead to the identification of novel targets for future vaccines.

In conclusion, we believe that *E. coli* disease in the chicken cannot be simply defined as being caused by a single pathotype of *E. coli*. In particular, colibacillosis is perhaps better defined as disease caused by *E. coli* rather than by Avian Pathogenic *E. coli*, and that the term APEC be reserved for the smaller number of well-defined "bona fide" pathogenic isolates with a range of defined virulence determinants that can reproduce disease in animal models. There are APEC, but not all disease-associated with *E. coli* in the chicken is caused by APEC.

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